Yeast Display scFv Antibody Library User's Manual

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Materials included:

NOTE: Before using the library, use the control scFv and antigen to become familiar with the processes of growth, induction, staining, and flow cytometry analysis. Do not use the library until you have used the control successfully. Your data should closely approximate Figures 4 and 6 for the control scFv. Before screeningn the library with your antigen, the library induction should resemble Figure 2 for HA and myc staining.

1. **EBY100.** 100-µL aliquot in 15% glycerol. Thaw rapidly at 30°C and streak on to YPD plate. Plate 30°C for 24 hours. Make your own freezer stocks.

2. **YVH10**. 100-µL aliquot in 15% glycerol. Thaw rapidly at 30°C and streak on to YPD plate. Plate 30°C for 24 hours. Make your own freezer stocks.

3. **pPNL6 plasmid.** 100-µL of 100 ng/mL. Transform into *E. coli* and select for Ampicillin resistant colonies. Isolate plasmid for use.

4. **pPNL9 plasmid**. 100-µL of 100 ng/mL. Transform into *E. coli* and select for Ampicillin-resistant colonies. Isolate plasmid for use.

5. **Control R3b-2 scFv in EBY100**. 100-µL aliquot in 15% glycerol. Thaw rapidly at 30°C and streak on to SDCAA plate. Plate 30°C for 48 hours. Make your own freezer stocks.

6. **Control biotinylated antigen**. 100-µL of 10 µM stock in PBS/1%BSA. Store at –20°C. Not to be used to select on library

7. **scFv library aliquot**. Approximately a 2-mL aliquot containing at least 10^{10} yeast. Thaw quickly at 30°C and resuspend in 100mls of YPD and incubate for 90 minutes 30°C to help the cells recover. (you should do a dilution plate at this point, dilution on YPD). Pellet cells and resuspend in 2 L of 2x- SDCAA media (see manual). Dilution = 1/2000 of 2-L culture, should give 500 colonies on plate. Grow plate at 30°C for 48 hours. Replica plate the colonies onto SDCAA plate. You will probably see about 2% of the colonies do not grow. Count colonies to determine starting titer of liquid culture. Grow liquid culture to saturation = 4-8 OD_{600}/mL, (24 to 48 hours) and freeze down aliquots representing 10^{10} cells for future use (2L x 4 OD/ml = 8000 OD mls which = 16 library aliquots (500 OD/aliquot). Our results show 1-1.5 x 10^{10} yeast grow on YPD plates and only 2-5% of these do not grow when replica plated onto SDCAA.

1. Introduction

This manual was created to help guide users of the scFv library described in an article published in the February 2003 issue of Nature Biotechnology titled "Flow cytometric isolation of human antibodies from a nonimmune *Saccharomyces cerevisiae* surface display library." The purpose of the manual is not to be all inclusive of every protocol associated with the potential uses of the library and subsequent clones, but to provide useful techniques, relevant references, and data figures demonstrating the employment of the described techniques. It is also clearly stated here and in the MTA the following:

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Creating affinity reagents to important biomolecules is one of the most critical, yet one of the most challenging tasks facing biologists. This chapter describes the implementation of a yeast surface display library of scFv (Single Chain Fragment Variable) antibodies as a method to solve part of this problem. The methodology was originally described by Feldhaus et al[1]. The scFv library is specifically designed to display full-length scFv antibodies whose expression on the yeast cell surface can be monitored with either C-terminal HA and N-terminal c-myc epitope tags. These epitope tags allow monitoring of clones or libraries of scFv clones for surface expression of full-length scFv by flow cytometry. The extra cellular surface display of scFv by *Saccharomyces cerevisiae* also allows the detection of appropriately labeled antigen-antibody interactions by flow cytometry. As a eukaryote, *S. cerevisiae* offers the advantage of post-translational modifications and processing of mammalian proteins and, therefore is well suited for expression of human derived antibody fragments. In addition, the short doubling time of *S. cerevisiae* allows the rapid analysis and isolation of antigen-specific scFv antibodies.

Yeast display, based on the platform created by Dane Wittrup at Massachusetts Institute of Technology, uses the a-agglutinin yeast adhesion receptor to display recombinant proteins on the surface of *S. cerevisiae*[2;3]. In *S. cerevisiae*, the a-agglutinin receptor acts as an adhesion molecule to stabilize cell-cell interactions and facilitate fusion between mating "a" and α haploid yeast cells. The receptor consists of two proteins, Aga1 and Aga2. Aga1 is secreted from the cell and becomes covalently attached to b-glucan in the extra cellular matrix of the yeast cell wall. Aga2 binds to Aga1 through two disulfide bonds, presumably in the golgi, and after secretion remains attached to the cell via Aga1. The yeast display system takes advantage of the association of Aga1 and Aga2 proteins to display a recombinant scFv on the yeast cell surface. The gene of interest is cloned into the pYD1 vector(InVitrogen), or a derivative of it, in frame with the *AGA2* gene. The resulting construct is transformed into the EBY100 *S. cerevisiae* strain

containing a chromosomal integrant of the *AGA1* gene. Expression of both the Aga2 fusion protein from pYD1 and the Aga1 protein in the EBY100 host strain is regulated by the *GAL1* promoter, a tightly regulated promoter that does not allow any detectable scFv expression in absence of galactose. Upon induction with galactose, the Aga1 protein and the Aga2 fusion protein associate within the secretory pathway, and the epitope-tagged scFv antibody is displayed on the cell surface (Figure 1a and b). Figure 2 shows the HA and c-myc epitope staining patterns for an scFv antibody library. Molecular interactions with the scFv antibody can be easily assayed by incubating the cells with a ligand of interest, Figure 1C. Figure 3 graphically depicts a generalized scheme for enriching and identifying antigen-specific binders within a non-immune scFv library. A combination of two rounds of selection using magnetic particles followed by two rounds of flow cytometric sorting will generally allow recovery of clones of interest.

Yeast surface display of scFv antibodies has also been successfully utilized to isolate higher affinity clones from small (~$1x10^6$) mutagenic libraries generated from a unique antigen binding scFv clone [4]. Mutagenic libraries are constructed by amplifying the parental scFv gene one wants to obtain higher affinity variants of using error-prone PCR to incorporate 3 to 7 point mutations/scFv[5;6]. The material is cloned into the surface expression vector using the endogenous homologous recombination system present in yeast, known as "Gap-Repair"[7]. Gap repair is an endogenous homologous recombination system in *S. cerevisiae* that allows gene insertion in chromosomes or plasmids at exact sites by utilizing as little as 30 base pair regions of homology between your gene of interest and its target site. This allows mutated libraries of 1 -10 x 10^6 clones to be rapidly generated and screened, in about 2 weeks, by selecting the brightest antigen binding fraction of the population using decreasing amounts of antigen relative to the KD of the starting parental clone. The screening involves 3 to 4 rounds of flow cytometry sorting, however, the flow cytometric sorting protocol is slightly different for a library based on a mutagenized clone than for a non-immune library and each will be described separately in section 3.4 and 3.6, respectively.

One of the real strengths of the yeast display system is the speed of characterizing the binding affinity of the clones[8]. A brief and greatly simplified version is described in this, Section 3.6.

Additional useful resources to complement the protocols in this Manual:
Yeast transformation: http://www.umanitoba.ca/faculties/medicine/biochem/gietz/Trafo.html

"*Saccharomyces cerevisiae*." Chapter 13, In *Current Protocols in Molecular Biology*. F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, K. Struhl, eds. John Wiley and Sons, Inc., New York (1993).

Methods in Enzymology, Volume 350: Guide To Yeast Genetics and Molecular Cell Biology, C. Guthrie and G. R. Fink, eds., Academic Press, San Diego, CA (2002).

2. Materials

2.1 Media and Agar Plates

In making the medias we add the following components in the following order, which appears to help with solubility of some components. Add amino acids to water, then the sugars, then add a 10x solution of buffer. pH should be ~6.25. We filter-sterilize all selective media. NOTE: YPED cultures saturate between 8 to 10 OD_{600}/mL, while SD + CAA cultures saturate between 3 to 5 OD_{600}/mL.

Rich Non-Selective Media: YEPD or YPD = Yeast Extract Peptone Dextrose

10 g/L yeast extract
20 g/L peptone
20 g/L dextrose
Filter sterilize or autoclave

Selective Growth = SD + CAA

5 g/L casamino acids (-ade, -ura, -trp)
20 g/L dextrose
1.7 g/L YNB (Yeast Nitrogen Base) w/out ammonium sulfate amino acids (Difco Becton Dickinson Difco YNB #233520)
5.3g/L ammonium sulfate
10.19 g/L Na_2HPO_4-$7H_2O$
8.56 g/L NaH_2PO_4-H_2
Note:
For 2x SDCAA we add 10g of casamino acids, and 3.4g of yeast nitrogen base,10.6g ammonium sulfate. This formulations can give greater cell numbers for growing the library up and freezing it down. Generally cell densities between 6-8OD/ml.

Selective scFv Induction = SG/R + CAA

Same as selective media except substitute the following sugars for Dextrose:
20 g/L galactose
20 g/L raffinose
1 g/L dextrose

Plates Buy or make them yourself: (TEKNOVA is our supplier)
YEPD same as above, but add 20g/L of Agar
SDA -HUT (Synthetic Defined Agar, -Histidine, -Uracil, -Tryptophan)

2.2 Strains & Plasmids

1. EBY100 (InVitrogen). (Leu^-, Trp^-) BJ5465 is MATa. It has the auxotrophic: ura3-52 (a Ty element insertion with no detectable background reversion frequency), trp1 (an amber point mutation), leu2δ200, his3δ200, pep4:HIS3, prbd1.6R, can1, GAL. EBY100 has genomic insertion of AGA1 regulated by GAL promoter with a URA3 selectable marker. The scFv library is displayed in this strain.

2. pPNL6 plasmid. Plasmid that our scFv library is cloned into as a fusion protein to AGA2. Selects for ability to grow in the absence of tryptophan in yeast and on ampicillin in E.coli.
3. YVH10 (**Ura-, Trp-**) is BJ5464 and is MAT-alpha. The other markers are: ura3-52 (a Ty element insertion with no detectable background reversion frequency), trp1 (an amber point mutation), leu2δ200, his3δ200, pep4:HIS3, prbd1.6R, can1, GAL. **YVH10** has the yeast PDI gene integrated in tandem with the endogenous copy and is under the constitutive glyceraldehydes (GADPH) promoter with Leu2 as a selectable marker. This strain is used to secrete and purify the scFv.

4. pPNL9 should be selected on SD + CAA + Trp . This is the plasmid we subclone our scFv into for secretion and purification. Induction media is the same as for pPNL6 except Trp is added.

2.3 Materials for Flow Cytometry and Magnetic bead enrichment

1. Miltenyi LS Macs columns and streptavidin magnetic beads (and anti-biotin magnetic beads)with manual magnetic separator.
2. *Anti-HA* (12CA5) antibody
3. Anti-c-myc antibody = 9E10 , 200ug/ml (Amersham) (pPNL6 derived libraries) or anti-V5 mAb (pYD1 libraries)
4. Goat anti-mouse(GaM) Alexa 488 conjugated (Molecular Probes)
5. Goat anti-mouse(GaM) Alexa 633 conjugated (Molecular Probes)
6. Goat anti-mouse (GaM)PE (Molecular Probes)
7. Streptavidin - R-phycoerythrin (SAPE) (Molecular Probes)
8. Streptavidin Alexa 633 (Molecular Probes)
9. NeutrAvidin Alexa 633 (Molecular Probes)
10. Biotinylated purified antigens, 2 μg/Kilodalton of antigen
11. Penicillin/Streptomycin (Gibco/BRL100x tissue culture grade)
12. Wash Buffer/Buffer = PBS + 0.5% BSA, 2 mM EDTA

3. Methods

The methods below are divided into 8 categories: 1) Growth and induction for scFv expression of libraries or clones, 2) Magnetic bead enrichment of biotinylated antigen binding yeast. 3) Fluorescent staining for both antigen binding and scFv expression of the library. 4) Flow cytometric sorting of library. 5) Clone validation and subsequent affinity determination. 6) Sorting of mutagenic scFv library for isolation of higher affinity clones. 7) Molecular protocols for analysis of clones and subcloning, and 8) secretion purification of scFv.

Many of the techniques, growth and induction conditions, and staining protocols are very similar between non-immune libraries, immune libraries, or mutagenized scFv clone libraries. The major differences between the various library types are both the diversity of the libraries (number of different antibody clones present) and the frequency of antigen binding clones within that diversity.

3.1 Growth and Induction of Surface Expression of scFv

NOTE: Cell numbers are generally determined by OD_{600} reading on a spectrophotometer. We find about 2 x 10^7 cells/mL per OD_{600} unit. This should be determined by plating yeast on agar plates or using a hemacytometer to calibrate your laboratories spectrophotometer.

3.1.1 Libraries of scFv:

1. Start a SD + CAA culture that contains a minimum of a 10x representation of the diversity of your library. For a 10^9 diverse library, 10^{10} yeast are needed to start the culture. The culture would be 1 L of 0.5 OD/mL. Plating a dilution plate is highly recommended to verify colony-forming units and lack of bacterial contamination.
2. Grow at 30°C with shaking overnight (baffle flask work best to increase aeration and increase growth). Take 10x representation of library diversity from culture and pellet. If you diversity is 10^9 clones, then a 10x representation would be 10^{10} yeast. That means at 2 x 10^7 yeast/OD_{600} unit you need 500 OD_{600}/mLs. For example, if your OD is 5.0 OD_{600}/ml then you need 100 mls of the culture to obtain 10^{10} yeast. Generally, the culture should be freshly saturated or be <4 $OD_{600/mL}$.
3. Resuspend the cell pellet in S/GR + CAA at 0.5 OD/mL and incubate at 20°C with shaking for 1 to 2 doublings as determined by OD. This will generally take 12 to 16 hours.
4. Wash the yeast once in wash buffer. The yeast are now ready to be labeled with biotinylated antigen and enriched on a magnetic column. You can also store the cells by placing the flask at 4°C for up to a week with minimal degradation of c-myc expression level or positive cells as determined by flow cytometry. However, the yeast do become sticky and tend to clump more, which may affect selections, and viability is often reduced by 50%.

3.1.2 Individual scFv Clones:

1. Start a 1- 5-mL SD +CAA culture from a single isolated yeast colony.
2. Grow at 30°C with shaking overnight.
3. Pellet enough cells to start a 1- 5-mL culture at 0.5 $OD_{600/ml}$ in induction media, SG/R + CAA.
4. Resuspend cell pellet in S/GR + CAA at 0.5 OD_{600}/mL and incubate at 20°C with shaking for 1 to 2 doublings as determined by OD_{600}. This will generally take 12 to 16 hours.
5. Wash cells once in wash buffer before staining for flow cytometry, or store the cells by placing the flask at 4°C for up to a week. See 3.1.1 for notes about 4°C storage of induced cultures. See section 3.5 for flow cytometric staining of individual clones for HA, Myc and antigen binding.

3.2 Magnetic Bead Enrichment Using Miltenyi Macs LS Columns for two rounds of magnetic bead enrichment.

Note: Biotinylated antigens can be generated in a variety of ways. We find the Pierce NHS Biotinylation kit, and the HABA system to quantify the number of biotin/molecule of protein, to be robust and easy. We strive to obtain 2-3 biotin/protein molecule. More biotin/protein is not desirable due to concerns about blocking the epitope/antibody interaction site.

3.2.1 Process for enrichment of antigen specific clones from a non-immune library of 10^9 diversity:

1. Resuspend 1 x 10^{10}yeast (from induced culture) in 10 mL wash buffer.
2. Add one or more biotinylated antigens at 100 nM final concentration. Incubate at 25°C (RT) for 30 minutes followed by or 5 to 10 minutes on ice. **All subsequent steps should be done with ice-cold buffer or at 4°C.**
3. Pellet cells (3000x g for 3 minutes) and wash 3x with 50 mL wash buffer.
4. Resuspend cell pellet in 5 mL wash buffer with 200 µL of Macs streptavidin magnetic micro beads (or anti-biotin magnetic micro beads) from Miltenyi. Alternating between these beads during subsequent rounds of selection decreases the chance of obtaining secondary reagent-specific clones.
5. Incubate on ice for 10 minutes with gentle mixing by inversion (?) every 2 minutes.
6. Pretreat Miltenyi LS column, loaded into magnet, by flowing 3 mL of ice-cold wash buffer through it using gravity.
7. Add 40 mL wash buffer, pellet cells and resuspend in 50 mL wash buffer. Make sure it is a single cell suspension by vortexing and passing cells through a cell strainer cap tube (Falcon Cat#352235) immediately prior to loading onto Miltenyi LS column.
8. Add the 7mls cell suspension that's was just put through the cell strainer cap to the column. After each 7 mL of cells have entered the column and the flow has stopped, remove the column from magnet and immediately put back into magnet. This rearranges the iron beads in the column and allows the cells that are physically trapped between the beads to pass through. With the column back in the magnet, add 1ml of wash buffer and let flow through, then another 7 mL of cells onto column. Repeat column removal procedure between each loading of cells. It will take about 30 minutes to load all 50 mL of cells.
9. Once all of the cells have been loaded on the column, wash the column with 3 mL of wash buffer. Make sure the upper loading chamber is washed of all residual cells. This wash removes the cells in the void volume of the column. Remove column from magnet and immediately replace as before. Repeat this wash 2 additional times.
10. Once the column has stopped dripping, remove the column from magnet and then add 7 mL of wash buffer and use the plunger to push all remaining cells out into a 15-mL conical tube. We generally elute about 1 - 3 x 10^7 cells. Pellet cells and resuspend as follows:

If Round 1 selection:

Resuspend yeast in 200 mL of selection media (SD + CAA with pen/strep). Plate a dilution to get accurate number of yeast eluted from the first column (This is the R1output diversity). Incubate culture to saturation,(10^{10} yeast). Approximately 24hours. Induce as above in SG/R + CAA. This allows maximal expansion of library and of your clone of interest for subsequent repeat of magnetic bead enrichment. Use anti-biotin magnetic beads (Miltenyi) as above for second round of selection. This is the R2output.

If Round 2 selection (second enrichment on magnetic column):

Resuspend cells eluted from the column in 500 µL buffer for subsequent staining for sorting by flow cytometry. Continue to Section 3.3

3.3 Fluorescent Staining of Cells from Macs Column Before Flow Cytometric Cell Sorting

It is important to note that at the start of each round of selection you should stain your library for anti-HA (12CA5)*, anti-c-myc (9e10), and secondary only controls. This establishes several important baselines. 1) Were my yeast properly induced? 2) What percent are expressing c-myc?(ie.full length scFv?) 3) Do my secondary reagents bind non-specifically? 4) Am I enriching for secondary reagent binders? Therefore, if you are going to use streptavidin-PE to label antigen bound cells, you should add just streptavidin-PE to your induced cells to see if it binds. The control for goat anti mouse reagents should be stained and analyzed (no anti-c-myc antibody should be added). We will generally see very little labeling of the aliquot, <0.1%. If you see labeling try other secondary reagents (i.e. Neutravidin, streptavidin or anti-biotin mAb) to minimize enriching for secondary reagent binders.*

NOTE: The following protocol is for staining up to 5 x 10^7 cells in 500 µL wash buffer. To stain less yeast, decrease the volume proportionally, yet keep the reagent concentration the same. Most secondary reagents should be tittered in your lab for the most appropriate dilutions. Figure 4 shows several combinations of secondary reagents that work well to label biotinylated antigens and anti-c-myc mAb.

1. Add 5 µL 9e10 anti-c-myc antibody (200ug/ml) to the 500 µL containing 1 -10 x 10^7 yeast eluted from the magnetic column.
2. Incubate for 30 minutes on ice. Pellet in microfuge on high for 10 seconds. Wash 2x with 500 µL buffer.
3. Add 1:200 dilution of secondary reagents (goat anti mouse –Alexa 488 and of streptavidin-PE). NOTE: It is important to use different secondary antigen-labeling reagents (streptavidin-Alexa 633 or neutravidin PE) between flow cytometric sorts to eliminate or reduce secondary reagent-specific scFv. Figure 4 shows several combinations of fluorescent reagents that can be used if secondary reagent binders are obvious.
4. Incubate 30 minutes on ice, pellet the cells, and wash 2x 500ul ice cold wash buffer.
5. Resuspend cells in 1 mL of buffer and keep on ice in the dark until sorting.

3.4 Flow cytometry sorting of antigen specific scFv clones from non-immune library

3.4.1 Sort gate decision:

Sorting from a non immune library that has gone through two rounds of enrichment on magnetic beads should allow you to see antigen binding yeast at a frequency of 1/1000 to 1/5000 cells. The sort gate is set in one of the following two manners.

The first method is based on sorting the top 0.1% of the brightest antigen binders that are also c-myc positive. These may or may not be an obvious or distinct population. Generally for the first sort (round 3 of selection), most or all of the cells coming off the magnetic column should be sorted. In figure 5, panel A, the population is obvious; however, this is not always the case (depending upon enrichment ratios) and sometimes antigen binding cells must be sorted on "faith". The second method relies on staining the same sub-library in the presence and absence of antigen. It is often very clear where to set the sort gate at this point. An example of sorting is presented in Figure 5.

The sort criteria are usually less stringent for the first flow cytometric sort. Sort more slowly (less coincidence aborts) and on an enrich mode. The rate of "more slowly" is dependent upon the sorter you are using. A skilled operator should be able to provide guidance.

3.4.2 First sort, Round 3 selection:

1. Sort yeast into eppendorf tubes containing 100 µL of YPD media. The YPD helps the yeast recover. We let them set in the YPD media for about an hour before growth on selectable media.
2. After sorting, plate the cells on SD+CAA Pen/Strep and an appropriate dilution plate. *Note: Yeast do not grow very well in liquid culture when grown at low cell densities ($<10^4$/ml). Therefore, when we sort out less than 10,000 yeast, we generally will plate them.*
3. Incubate plates at 30°C for 24 to 48 hours.
4. Scrape colonies together and then grow for several hours in SD+ CAA media.
5. Subculture at least a10x representation of sub-library diversity (determined from the dilution plate of sorted cells) into S/GR + CAA induction media.
6. Make glycerol stock of at least 10x representation of the sub-library diversity from the SD+CAA culture for storage in case subsequent steps need to be repeated. The glycerol stock is the yeast in a 15% glycerol solution and can be stored at –80°C. The cell concentration in the glycerol stock can be from 1 OD_{600}/ml up to 100 OD_{600}/ml.

3.4.3 Second Sort, Round 4 selection: At this point your diversity is generally under 100,000. Therefore, staining 2 x 10^7 yeast gives you a 200 fold coverage of your diversity. You also expect to see clones that bind your antigen at a frequency of 1-10%. Cells are stained as before, with all controls:

Control 1 = Unstained
Control 2 = GaM488 only
Control 3 = SA-PE only
Control 4 = anti-myc + GaM488
Control 5 = anti-myc + GaM488 + SAPE
Sample = anti-myc/GaM488 + Antigen/SAPE

A number of controls (listed above) should be done with the induced R3 sub-library output prior to the 4th round of selection. Control #5 will help distinguish antigen detection reagents specific binders from true antigen binders. Analyze at least 100,000 events to allow patterns to emerge in the bivariate plot. SA-PE positive clones that do not have the c-myc tag can be easily removed during the subsequent sort by setting the selection gate to isolate only c-myc positive/antigen binding cells.

If the SA-PE positive clones from control #5 are also c-myc positive, then careful examination of control #5 to the antigen containing sample may allow a different subpopulations to be visualized in the dual positive quadrant of the bivariate plot. The population that is present in the antigen containing sample and not in the no antigen control, can be specifically isolated. However, you may also see the percent dual positive population change from 2 % in the no antigen control to 4% or more in the antigen containing sample. These antigen binder are able to be identified in one of two manners. First, after sorting with SA-PE, individual clones can be subsequently screened for antigen binding specificity. The number of clones to analyze will be dictated on the

relative differences in the percent positive between control #5 and the antigen containing sample. Second, the secondary reagent used to detect antigen positive cells can be changed to anti-biotin mAb or neutravidin, both labeled with an appropriate fluorescent dye. This prevents those clones in the library that specifically bind streptavidin from contaminating the selection process altogether. However, make sure to repeat control #5 with whatever secondary reagent you intend to use to detect antigen binding.

1. Sort on "purity mode" if an antigen specific population is obvious. Sorting a few hundred to a thousand yeast cells at this point will generally suffice to give an antigen positive clone.
2. Sort yeast into eppendorf tubes containing 100 µL of YPD media.
3. After sorting, plate the cells on SD + CAA Pen/Strep. No dilution plate is required when less than 1000 yeast are plated. We generally see a 50-80% plating efficiency.
4. Incubate plates at 30^{o}C for 24 to 48 hours.
5. Pick individual colonies into SD + CAA media. We will generally pick 10 different clones. These may all be the same or ten unique clones. The number of unique clones will be determine either by sequencing or BstN1 finger printing of PCR amplified scFv genes[9].
6. Subculture 100ul into 1ml of SGR + CAA. Grow at 20^{o}C for 16 hours to induce expression of scFv.

3.5 Clone validation and affinity determination.

This verifies the individual clones from the previous sort are true antigen binders and that they express full length scFv as determined by myc expression. Each sample needs to be stained with antigen and without antigen.

No Ag = anti-myc + GaM488 + SAPE

Plus Antigen = anti-myc/GaM488 + Antigen/SAPE

3.5.1 Flow cytometric staining of individual clones:

1. Prepare 1-2 x10^{6} yeast by washing the induced yeast 1x with 1ml of buffer.
2. Resuspend yeast in 100ul containing 1ul of anti-c-myc mAb and for plus antigen stain biotinylated antigen (generally 100nM)
3. Incubate for 30 minutes at room temperature followed by 5 minutes on ice. Pellet in microfuge on high for 10 seconds. Wash 2x with 500 µL of ice cold buffer.
4. Resuspend pellet in 100ul of 1/200 dilution of secondary reagents (goat anti mouse – Alexa 488 and of streptavidin-PE). Note: Figure 6 shows several combinations of fluorescent reagents that can be used.
5. Incubate 30 minutes on ice, pellet the cells, and wash 2X in cold buffer.
6. Resuspend cells in 1 mL of buffer and keep on ice in the dark analysis by flow cytometry.

3.5.2 Affinity determination using equilibrium binding titration curves to determine equilibrium dissociation constants K_D.

Due to the initial selections being done at 100nM, the vast majority of clones identified from a non-immune library will be in the 1nM-100nM affinity range. The lower the concentration of antigen in the initial selection, the lower the affinity range will be and fewer unique clones are generally isolated. Once antigen binding has been verified, we

determine clone uniqueness by utilizing a DNA fingerprint of the PCR amplified scFv restriction digested with BstN1[9;10]. This limits the number of clones we need to determine the K_D for, as many clones will may be identical as demonstrated by identical BstN1 fingerprints obtained above. This range of affinities can be determined on the yeast surface by measuring the amount of antigen bound at different concentration at equilibrium. The technique relies on measuring the mean fluorescence intensity (MFI) of the bound antigen, at and variety of concentrations of antigen, on the c-myc positive yeast. K_D is measured by determining at what concentration of antigen is half of the scFv on the surface of the yeast cell bound to antigen. Therefore measuring the MFI of the yeast when no antigen is bound and determining the concentration of antigen that gives the maximal MFI is needed. This is easily accomplished by setting up a series of antigen concentrations in which to label the yeast with and then measuring the MFI of the antigen binding population by flow cytometry. The MFI, obtained using flow cytometry with each of the antigen concentrations tested, is then plotted against the antigen concentration and using a nonlinear least squares curve to fit the data, the K_D is determined. See Figures 6 & 7.

Staining 10^5 yeast/antigen concentration represents approximately 10^9 to 10^{10} antigen binding sites (scFv) in the sample. We assume 10^5 scFv/yeast and 50% express scFv. You want to maintain at least 10x excess of number of molecules of antigen over the number of scFv molecules. This is non-depleting ligand conditions.

We routinely prepare 12 clones using a 96 well micro titer plate format choosing 8 concentrations of antigen, which include a "no antigen" control for background.

1. Label 10^6 induced yeast with 100ul of a 1/100 dilution on the c-myc mAb(200ug/ml) for 30minutes on ice.
2. Add 10ul (10^5 yeast) directly into the antigen concentrations (see step 3 below). This uses very little c-myc antibody, 1ul/100ul of 10^6cells.
3. The following concentrations and volumes will allow for an accurate K_d to be measured for affinities between 1nM –100nM. 10^5 yeast/sample = 5 x 10^9 scFv. If you have an idea of what your affinity is you can omit the first 2 or last antigen concentrations. For example, 100nM affinity you do not need the 1 and 0.5nM antigen concentrations. For a 5nM affinity you do not need the 500nM or 100nM concentrations. You are striving to do a series of 2-5 fold dilutions at 5-10x over K_d to 5-10x below K_d.

Concentration of Antigen	Volume of antigen solution	# of molecules of antigen
500nM	100ul	3 x 10^{13}
100nM	100ul	6 x 10^{12}
50nM	100ul	3 x 10^{12}
25nm	100ul	1.5 x 10^{12}
10nM	100ul	6 x 10^{11}
5nM	100ul	3 x 10^{11}
1nM	500ul	3 x 10^{11}
0.5nM	500ul	1.5 x 10^{11}= 30x excess
0.1nM	1ml	6 x 10^{10} = 10x excess

4. Incubate the samples at room temperature for 30 minutes. This allows ample time for the binding reaction to come to equilibrium. The system comes to equilibrium within a couple of minutes at a concentration of 1 log above or below the concentration at K_D.
5. Put samples on ice for 5 minutes. The decrease in temperature greatly decreases the off rate. For a 25^{o}C decrease in temperature a 50-100 fold decrease in off rate is seen [11].
6. Wash sample 2x with 500ul ice cold buffer.
7. Resuspend sample in 100ul of a 1/200 dilution of secondary reagents goat anti-mouse-–Alexa 488 and of streptavidin-PE.
8. Incubate on ice for 30 minutes. Pellet cells and wash 1x with 500ul ice cold buffer.
9. Resuspend in 500ul of buffer and store on ice in dark until analyzed by flow cytometry.
10. Gather 10,000 events for a bivariate plot using the fluorescence channels that look at Alexa 488 (Emission 525, usually FL1 on Becton Dickinson and Coulter Brand flow cytometers) and Phycoerythrin (Emission 550nm FL2 on Becton Dickinson and Coulter Brand flow cytometers).
11. Collect the statistical information from the dual c-myc+ /antigen+ population.
12. Plot the MFI against the concentration of antigen and use a nonlinear least squares to fit the curve (such as found in Kaleidgraph or GraphPad) and determine the K_D using the following equation:

$$y = m1 + m2* m0/(m3 + m0)$$

where y = MFI at given antigen concentration, m0 = Antigen concentration, m1 = MFI of no antigen control, m2 = MFI at saturation, and m3 = K_D. If "R" values are generated, values of 0.998 and greater will give K_D values accurate within 30%.

3.6 Sorting of mutagenic scFv library for isolation of higher affinity clones.

Boder *et al*. developed a methodology for generation and isolating higher affinity mutants of a specific scFv. This was demonstrated by the maturation from nanomolar to femtomolar binding K_D for an scFv [4]. The construction of a mutagenized library can be done several ways and will not be covered here[6;12;13]. However, there are several guidelines one should follow. The number of mutations per clone will affect the number of mutants that can display function full length scFv. The number of mutations per clone will also affect the likely hood of finding a clone of increased affinity[5]. A library of 10^6 to 10^7 different mutants can easily be screened using the methodology below.

Protocol for higher affinity clone isolation is as follows:

1. Grow and induce library with a minimum of 10x coverage.
2. Stain no more than 10^8 cells (takes about 3 hours to sort at 10,000 events/sec).
3. Label yeast with anti-c-myc to identify full length scFv antibody clones. The mutagenesis will induce a larger number of stops and truncated proteins. At the same time, add antigen at the K_D of the parental clone. Incubate for 30 minutes at room

temperature followed by 5 minutes on ice before centrifugation and washing cells twice.

4. Resuspend cell pellet in secondary reagents. Incubate on ice in the dark for 30 minutes. Wash once and resuspend.
5. Run sample on sorter. Sort entire sample for the brightest 1.0% of c-myc-positive antigen-binding population. Expect 5% to 20% of the cells to express c-myc-positive scFv, and of these 10% to 20% will bind antigen.
6. Cells will either be plated on selective plates that contain antibiotics or grown in selective liquid media (about 5 mL SD+CAA). The antibiotics can be any or all of the following: pen/strep 10 mg/mL used at 100 µg/mL, ampicillin, kanamycin 10 mg/mL used at 10 to 20 µg/mL.
7. Plates are incubated at 30^{o}C for 1 to 2 days. Colonies are pooled and then induced. Liquid media grown cells are centrifugation and induced. An aliquot is generally frozen as well.
8. Therefore, from 10^7 cells you might sort out a total of 10^3 to 10^4 cells. Typically, 50% of the cells will form colonies or will be viable. For subsequent sorts, 70% of the cells will be c-myc-positive, with the vast majority being binding antigen.
9. After regrowth and induction of the yeast from the Round 1 selection, 10^8 are labeled with antigen at a concentration of 0.5X to 0.1X the concentration at K_D. The addition of anti c-myc and the staining with secondary reagents is as described in section 3.5.
10. 10. Sort on the brightest 0.1% of antigen-binding c-myc positive clones. Note: The sub-libraries overall K_D can be determined using a similar approach as determining the K_D of an individual clone as outlined in section 3.5. We will generally do our first sort with antigen concentration at Kd and then drop the concentration by 2 – 10 fold each subsequent sort.
11. Repeat steps 6-10 for 2 additional rounds.
12. Individual clones can be assayed for K_D and sequencing of the insert can determine number of unique clones and where particular changes from the parental clone occur. Generally, by the forth round of selection 80% of the clones in the sub-library will be improved mutants.

Note: We often place a sort gate in the are where a saturated clone resides in the bivariate plot for the parental clone. The sorts use 1/10th antigen concentration at Kd for the parental, thus the parental clone is no longer present in the sort gate window. One may be sorting at a frequency of less than 1/10,000 and only sort out 1000 yeast. However, in two selection cycles (less than 1 week time) we routinely obtain clones with improved affinity.

It is often useful to run the original clone side by side with your new mutants to verify even slight differences in K_D, 3 fold differences in K_D are easily seen.

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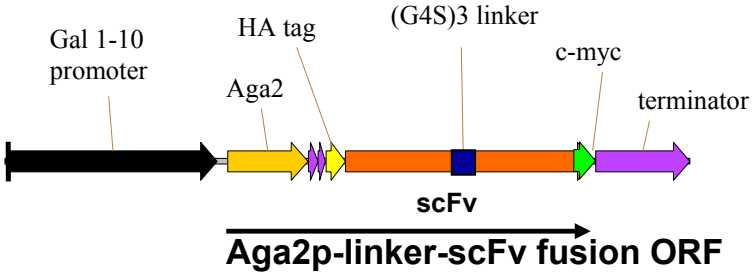
1. Features of the pPNL6 Vector

The pPNL6 vector containing the scFv library offers several key features that make it easy to display proteins of interest on *S. cerevisiae*. These include:
GAL1 promoter for strong inducible expression following the addition of galactose
N-terminal HA and C-terminal c-myc epitopes for simplified detection of the displayed scFv antibody with an Anti-c-myc (9e10) or Anti-HA(12CA5) Antibody
TRP1 auxotrophic marker and *CEN/ARS* origin for selection and maintenance in *S. cerevisiae*

Figure 1. Display of scFv Antibody Library Using pPNL6

A. The GAL1/10-regulated scFv surface expression construct.



B. The scFv Aga2 fusion protein surface expression.

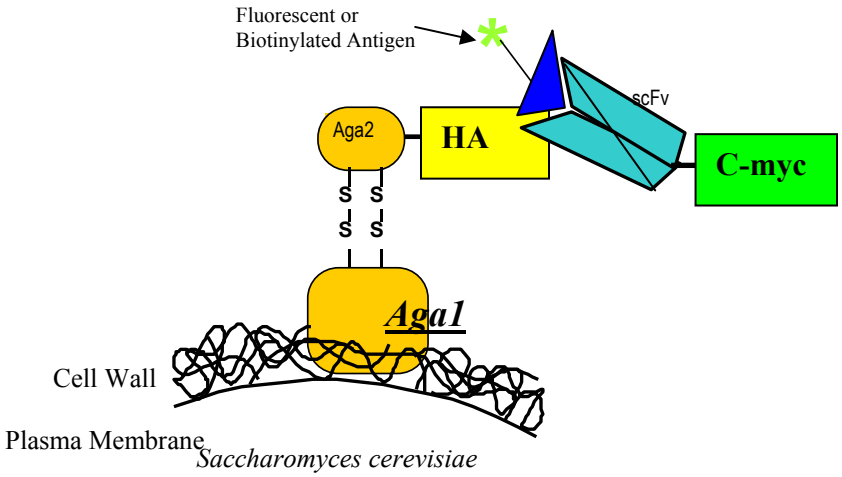


Figure 2. Flow cytometric analysis of scFv library expression on yeast. The red histogram represents the background auto-fluorescence. The blue histogram represents the HA epitope tag positive cells. 60 to 70% of the yeast in the scFv library will be HA epitope tag positive. The green histogram represents the c-myc expressing yeast and represents 35-40% of the total.

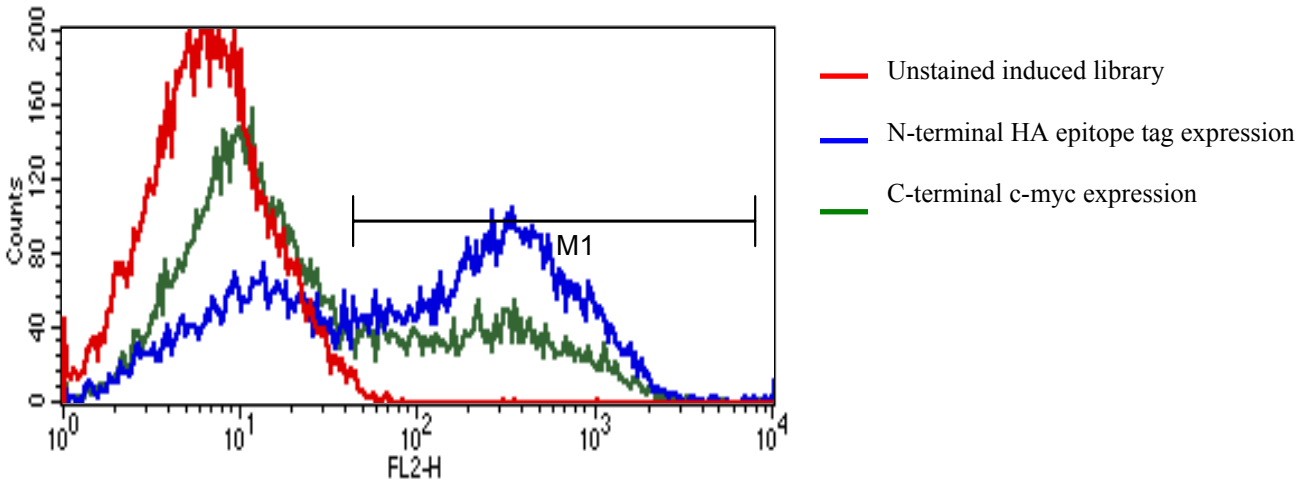
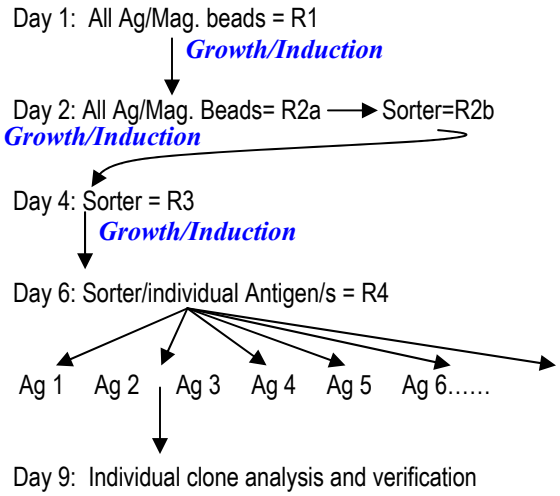


Figure 3. Isolation of scFv through sequential enrichment
A. Schematic of generalized scFv library screen.

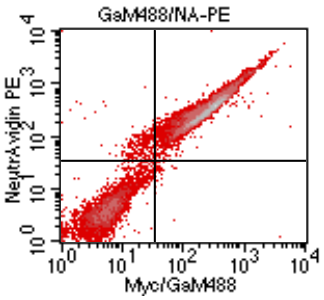


B. Enrichment factor and recovery of antigen-specific clones using a combination of magnetic bead enrichment and flow cytometric cell sorting. It is important to use at least 10-fold coverage of the starting complexity library to fully screen the library. After two rounds of enriching using magnetic beads the 500 Ag-specific cells would theoretically be present in a total of 5 x 10^7 non-specific cells. Using the Miltenyi Macs system described in this manual, we find that from 10^{10} cells loaded onto the column, the background is consistently around 0.5 to 5 x 10^7 cells (based on OD_{600}), regardless of scFv expression or number of antigen-binding cells. The cell sorter greatly reduces the complexity from 10^7 to 10^4 to 10^5 total cells depending on the stringency of sort gates and total number of cells sorted. By the second or third round of flow cytometric sorting, it's usually apparent if an Ag-specific clone is present at a frequency greater than 1/10,000. It is always important to have a sample prepared that has been stained with all secondary regents, but without antigen (i.e., anti-c-myc/GaM-PE and streptavidin-Alexa 633).

Selection Round and type	Complexity	Clone Frequency = Ag-specific/ complexity	Library coverage screened	Ag-specific cells/Total cells	Recovery of Ag-specific cells based on 50% recovery per round of selection	Selection output	**Enrichment =** Original Complexity x Output Complexity
R1 Magnetic beads	10^9	$1/10^9$	10x	$10/10^{10}$	5	1 x 10^7	$1/10^9$ x $5/10^7$ =500x
R2 Magnetic beads	1 x 10^7	$5/10^7$	1000x	$1000/10^{10}$	500	1 x 10^7	$5/10^7$ x $5/10^5$ =500x
R3 Cell sorter	1 x 10^7	$5/10^5$	0	500/1 x10^7	250	1 x 10^4	$5/10^5$ x $2.5/10^2$ =2000x
R4 Cell sorter	250 x 10^4	2.5/100	1000x	$25{,}000/10^6$	12500	1000	$2.5/10^2$ x 1000/1000 to 1000/2000 about 50x
R4output sorter-analysis	5 x 10^4	5 to 9/10					

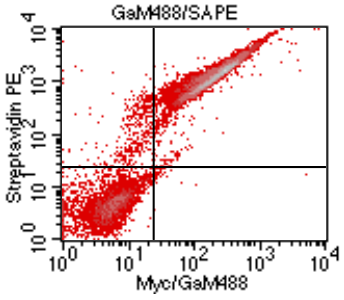
Figure 4. Different fluorescent reagents that can be employed to detect c-myc expression and biotinylated antigen binding. Panel A. Bivariate flowcytometric analysis. Panel B. Comparison of MFI (Mean Fluorescence Intensity) is shown.

030122: Analysis of different detection reagents for antigen and Myc tag.
scFv 378 R3b2 clone used for analysis



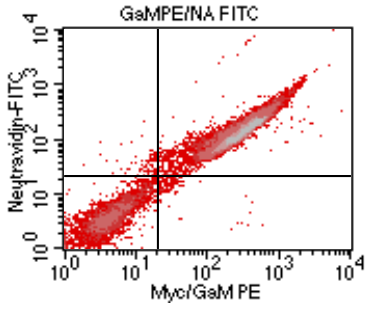
Sample ID: GaM488/NA-PE
Acquisition Date: 22-Jan-03
Total Events: 20000
X Parameter: FL1-H Myc/GaM488 (Lo
Y Parameter: FL2-H NeutrAvidin PE (L

Quad	% Total	X Mean	Y Mean
UL	1.58	21.76	111.83
UR	77.33	282.69	374.02
LL	20.94	6.61	5.35
LR	0.14	168.10	21.24



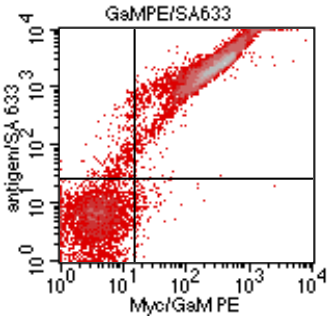
Sample ID: GaM488/SAPE
Acquisition Date: 22-Jan-03
Total Events: 20000
X Parameter: FL1-H Myc/GaM488 (Log)
Y Parameter: FL2-H Streptavidin PE (Lo

Quad	% Total	X Mean	Y Mean
UL	2.11	13.76	217.47
UR	76.73	234.55	1384.01
LL	21.04	5.96	5.59
LR	0.10	249.55	17.69



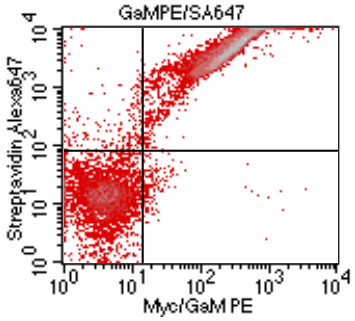
Sample ID: GaMPE/NA FITC
Acquisition Date: 22-Jan-03
Total Events: 20000
X Parameter: FL2-H Myc/GaM PE (Log)
Y Parameter: FL1-H Neutravidin-FITC (Log

Quad	% Total	X Mean	Y Mean
UL	0.65	15.02	36.80
UR	77.09	319.88	161.59
LL	21.81	5.02	4.58
LR	0.46	75.22	12.91



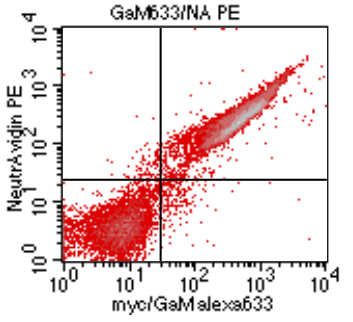
Sample ID: GaMPE/SA633
Acquisition Date: 22-Jan-03
Total Events: 20000
X Parameter: FL2-H Myc/GaM PE (Log)
Y Parameter: FL4-H antigen/SA 633 (Lo

Quad	% Total	X Mean	Y Mean
UL	1.93	9.27	142.58
UR	77.75	310.47	2521.77
LL	19.71	4.22	6.43
LR	0.60	43.96	10.68



Sample ID: GaMPE/SA647
Acquisition Date: 22-Jan-03
Total Events: 20000
X Parameter: FL2-H Myc/GaM PE (Lo
Y Parameter: FL4-H Streptavidin Alexa

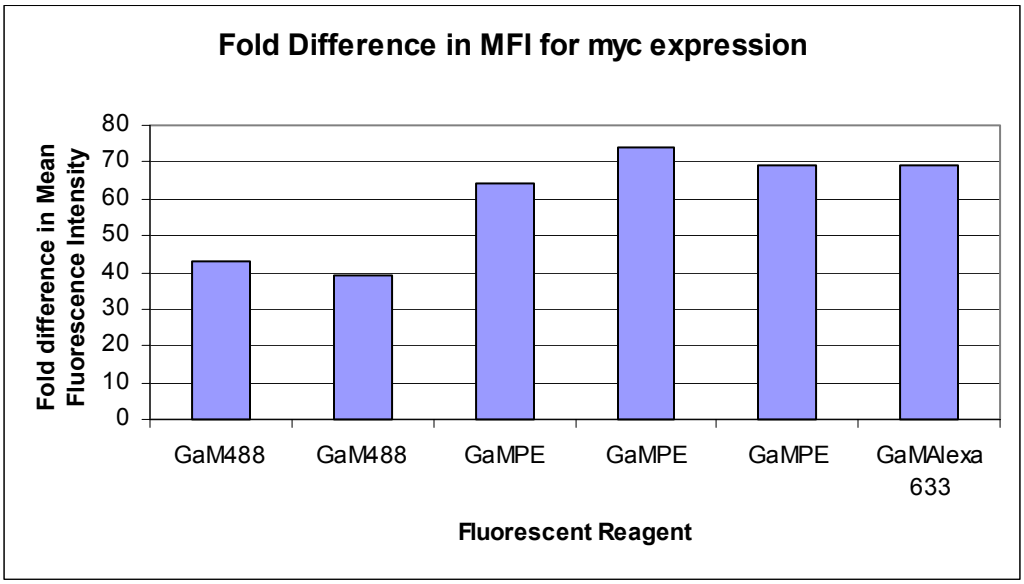
Quad	% Total	X Mean	Y Mean
UL	1.28	7.36	602.13
UR	77.37	310.67	4801.63
LL	20.65	4.46	16.41
LR	0.70	107.39	25.90



Sample ID: GaM633/NA PE
Acquisition Date: 22-Jan-03
Total Events: 20000
X Parameter: FL4-H myc/GaM alexa633 (Lo
Y Parameter: FL2-H NeutrAvidin PE (Log)

Quad	% Total	X Mean	Y Mean
UL	0.60	19.71	75.69
UR	76.70	554.27	330.34
LL	21.79	7.98	4.61
LR	0.92	1319.72	11.27

Panel B. Fold difference of myc expression and antigen binding using different detection reagents over background Mean Fluorescence Intensity (MFI) of yeast auto fluorescence.



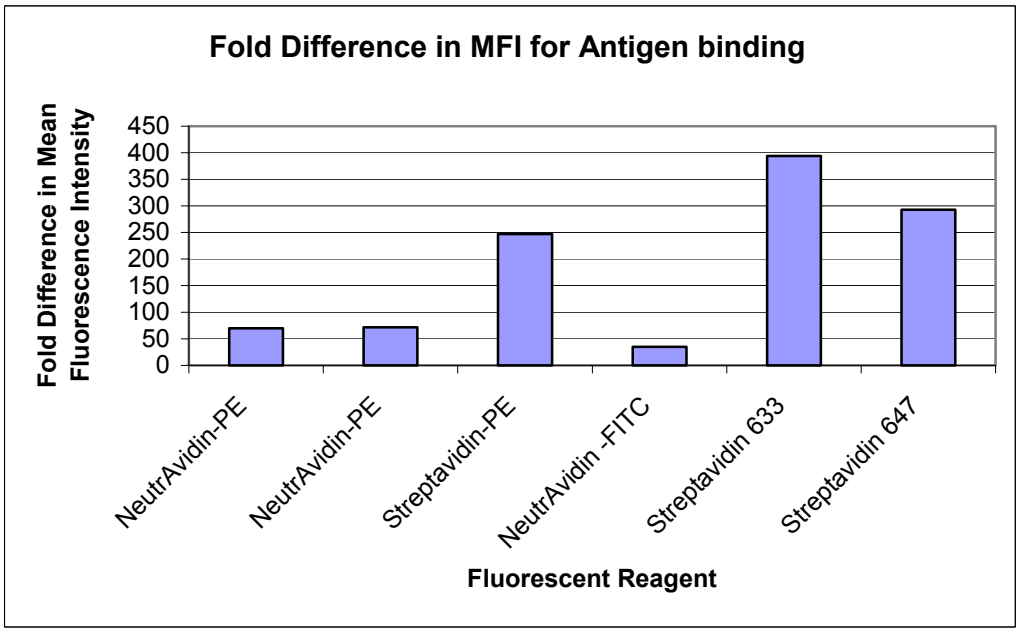
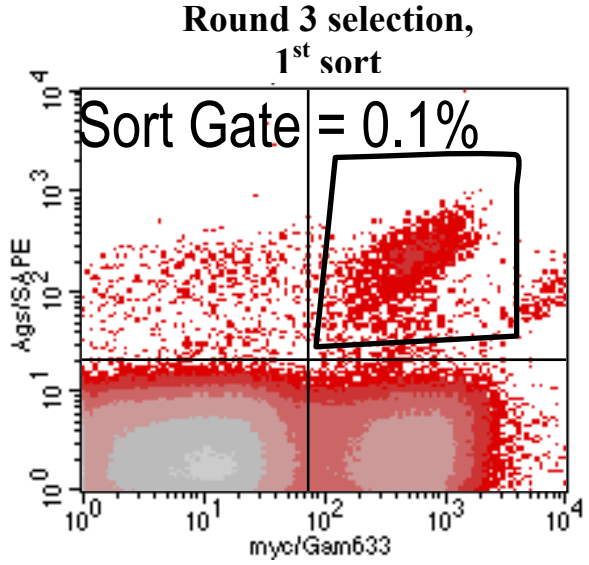


Figure 5. Sorting of R2 output from second magnetic bead enrichment column to create R3 output and then R4 output. The "no antigen" control shows that when antigen is present there are many more dual positive yeast and provides confidence that what you are sorting is actually binding your antigen and not the detection reagent.



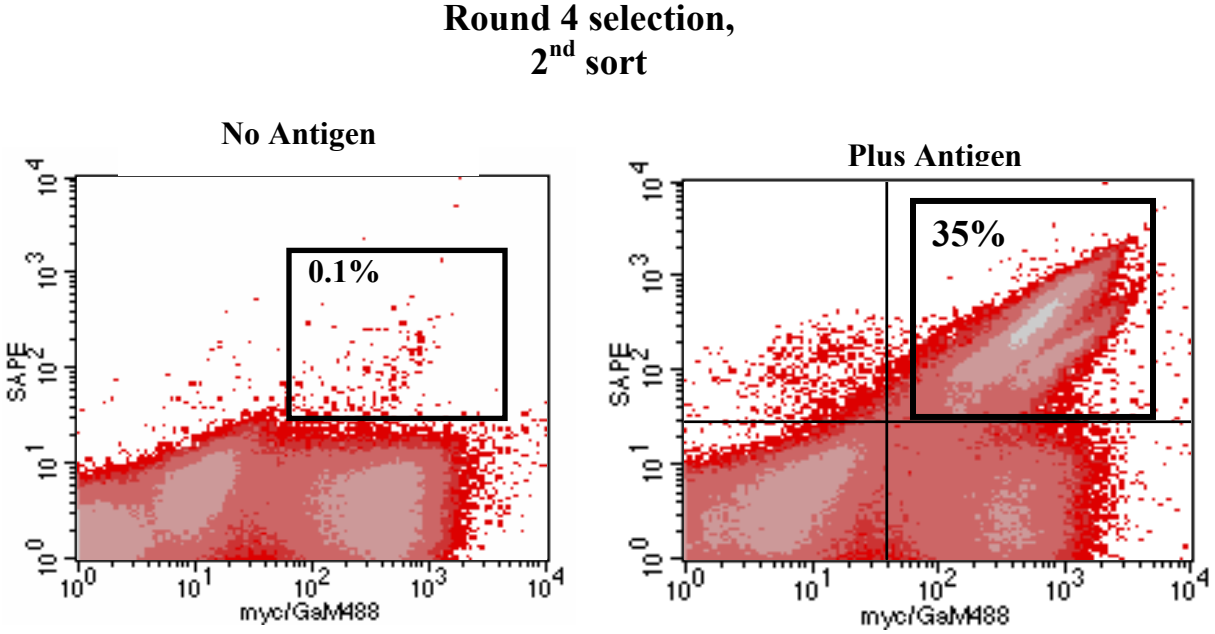
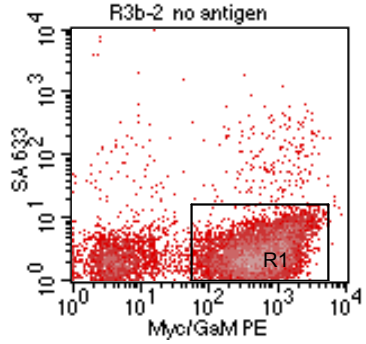
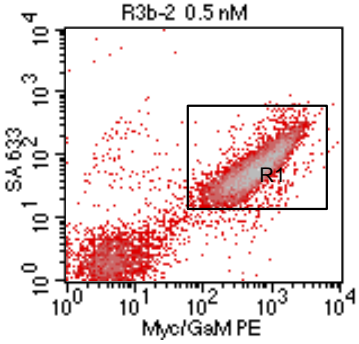


Figure 6. K_D determination using flow cytometry



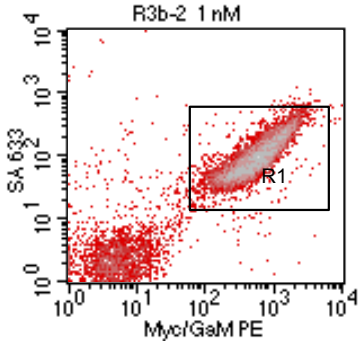
File: R3b-2 no antigen
Total Events: 10000

Region	% Total	X Mean	Y Mean
R1	75.89	712.29	3.50



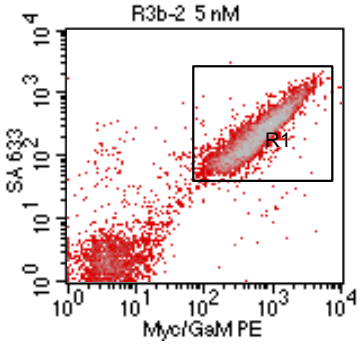
File: R3b-2 0.5 nM
Total Events: 10000

Region	% Total	X Mean	Y Mean
R1	74.07	689.25	77.37



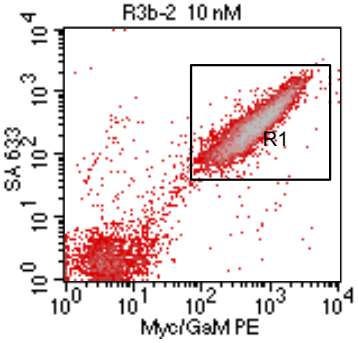
File: R3b-2 1 nM
Total Events: 10000

Region	% Total	X Mean	Y Mean
R1	77.92	661.16	121.76



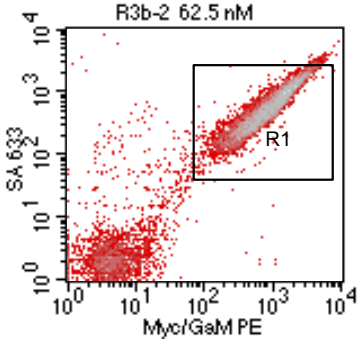
File: R3b-2 5 nM
Total Events: 10000

Region	% Total	X Mean	Y Mean
R1	78.32	759.32	294.88



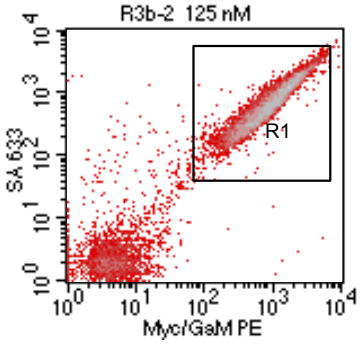
File: R3b-2 10 nM
Total Events: 10000

Region	% Total	X Mean	Y Mean
R1	78.57	715.84	376.33



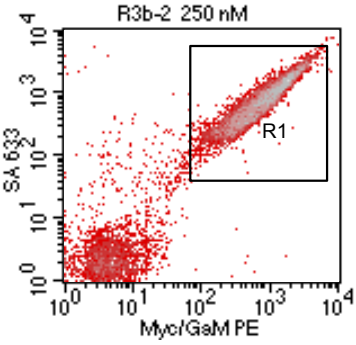
File: R3b-2 62.5 nM
Total Events: 10000

Region	% Total	X Mean	Y Mean
R1	75.92	982.43	687.89



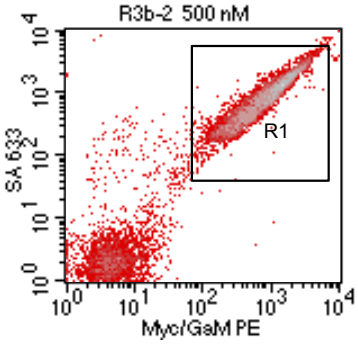
File: R3b-2 125 nM
Total Events: 10000

Region	% Total	X Mean	Y Mean
R1	77.58	1042.96	839.12



File: R3b-2 250 nM
Total Events: 10000

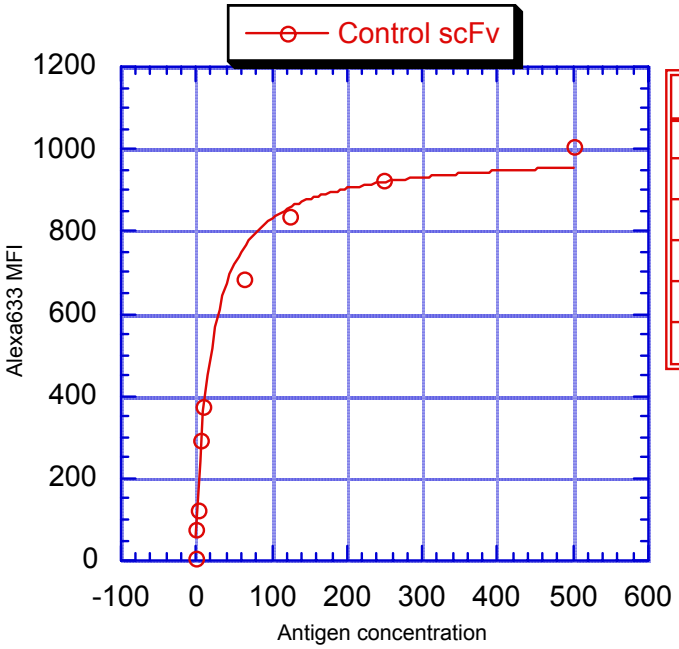
Region	% Total	X Mean	Y Mean
R1	76.25	925.85	924.46



File: R3b-2 500 nM
Total Events: 10000

Region	% Total	X Mean	Y Mean
R1	73.53	934.21	1004.98

Figure 7. K_D determination using flow cytometry and nonlinear least squares fit



y = m1 + m2*M0/(m3+m0)		
	Value	Error
m1	59.065	30.611
m2	934.13	43.673
m3	21.096	5.2127
Chisq	14715	NA
R	0.99389	NA

Antigen concentration	Alexa633 MFI
0.0000	3.5000
0.50000	77.370
1.0000	121.70
5.0000	294.90
10.000	376.30
62.500	687.90
125.00	839.10
250.00	924.50
500.00	1005.0

3.7 Molecular protocols for analysis of clones and subcloning

Primers for PCR Amplification and Sequencing of scFv

NOTE: Anytime you are going to amplify and subclone an scFv it is very important to use Platinum Tag HiFi or another proofreading thermal-stable polymerase. Do NOT use HiFi for mutagenic PCR.

PCR Amplification of scFv from PNL6 surface expression vector

PNL6 Forward: GTACGAGCTAAAAGTACAGTG
PNL6 Reverse: TAGATACCCATACGACGTTC

Note: The primers above, (PNL6 for and rev), will not hybridize or amplify a segment of DNA from the starting pPNL6 vector. The primers anneal to part of the cloned scFv gene and to part of the starting vector. Therefore, only scFv library clones will be amplified.

PCR amplification of scFv for "GAP Repair" into PNL9 secretion vector

It works best if the template is either purified plasmid or PNL6for/rev amplicons. The underlined area is the homology between the PNL6 amplicon and the primer; i.e., the primer-binding site. You can also amplify from a genomic prep. Use HiFi Tag (a proofreading enzyme).

*PNL9for: GACGTTCCAG ACTACG*CTGG TGGTGGTGGT TCTGCTA
PNL9rev: GGGTTAGGGA TAGGCTTACC CTGTTGTTCT AGAATTCCG

PNL9/Secretion vector colony PCR of scFv insert for sequencing or +/- insert check

Gal1 for: AATATACCTCTATACTTTAACGTC
CYC1 rev: GCGTGAATGTAAGCGTGAC
Gal1b (for sequencing primer): AATATACCTCTATACTTTAACGTCAAGG

For sequencing PCR amplicons from PNL6 and PNL9

Long Reads (PNL6 only):

Rev Seq P2: CCG CCG AGC TAT TAC AAG TC
For Seq P2: TCT GCA GGC TAG TGG TGG TG

NOTE: This sequencing primer can read all the way through the heavy and light chain without having to reverse-transcribe the sequencing information before querying the Vbase data base (http://www.mrc-cpe.cam.ac.uk/vbase-ok.php) to determine the heavy- and light-chain usage. However, you must put in only half the sequence at a time.

Short Reads (PNL6 or PNL9):

These are useful for sequencing of colony PCR of gap-repaired scFv into secretion vector or surface expression vector.

G4SL: GTT CCG GAG GCG GCG GTT C
G4SH: AAC CAC CAC CGC CGC TGC C

3.7.2 PCR Cycle Conditions for different Reactions using different templates

A) Purified Plasmid pPNL6 based scFv amplification
Primers:
PNL6 Forward: GTACGAGCTAAAAGTACAGTG
PNL6 Reverse: TAGATACCCATACGACGTTC

Standard PCR Conditions for pNL6 for and rev Primer Pairs using MJ DNA Engine			
	Stock Conc	µL/reaction	Conc. in reaction
10 x Buffer	10x	5	
$MgCl_2$	50 mM	1.5	1.5 mM
dNTPs	10 mM	1	0.2 mM
H_2O		39-X	
Primers	10 µM each	2	0.2 mM each
Template		X	
Platinum Taq (Gibco)	5U/µL	0.5	
Cycle: 95°C 5 minutes, 95°C 30 seconds, 50°C 30 seconds, 72°C 45 seconds, repeat 29 or 34 cycles, 72°C 5 minutes.			

Note: These primers (PNL6 for and rev) will not hybridize or amplify a segment of DNA from the starting pPNL6 vector. The primers anneal to part of the cloned scFv gene and to part of the starting vector. Therefore only scFv library clones will be amplified.

B) Yeast Colony PCR

Primers:

PNL6 Forward: GTACGAGCTAAAAGTACAGTG

PNL6 Reverse: TAGATACCCATACGACGTTC

Prepare microfuge tubes(or PCR tubes) containing 20 µL 0.1% SDS

Use pipette tip to transfer yeast colony to tube (or pellet 100 µL of cells from a 1- to 3-day-old liquid culture and then add 20 µL 0.1% SDS). Try for 1 x 10^7 cells, a medium-sized colony. Err toward having too much as opposed to not having enough. Pipette up and down. Vortex on high for ~10 seconds.

Heat at 95°C for 5 minutes, spin (30 seconds on high), and add 1 to 2 µL of supernatant into 50 µL PCR reaction. (Typically, the samples are heated in an MJ thermocycler.)

Reaction and conditions (see below):

OTHER NOTES: Cell age is critical. Plates are 1 to 7 days old. Anything more than 7 days is far less successful. You can also use a broth culture; just spin down enough cells to see a small pellet (approximately the size of a colony), discard supernatant (wash with water), and proceed like a colony PCR. Once the cells have been lysed, keep them on ice; they can be used successfully for a few hours if kept on ice. However, we have had little (or no) success using the lysed cells 1 day later.

Yield: Typical yield is ~20 to 25 µg/µL (Total of 800 ng to 1 µg for a 40-µL reaction). We typically load 3 or 4 µL on a gel to check concentration and size. This is enough material to do a BstNI digest (See Below for fingerprint analysis) and sequencing with G4SH and G4SL primers. If you are just looking for "positives" for inserts, cut the volumes in half.

Yeast Colony PCR Conditions			
	Stock Conc.	µL /reaction	Conc. in reaction
10 x Buffer	10x	5	1x
$MgCl_2$	50 mM	1.5	1.5 mM
dNTPs	10 mM	1	0.2 mM
H_2O		37-X	
Primers	10 µM each	2	0.2 mM each
Template		X	
10% triton-x100		2µL	
Platinum Taq (Gibco)	5U/µL	0.5	
Cycle: 95°C 5 minutes, 95°C 30 seconds, 50°C 30 seconds, 72°C 45 seconds, cycle 30x, 72°C 5 minutes.			

NOTE: Increase the annealing temperature by 5°C when using genomic DNA or plasmid template in PCR reactions.

C. PCR conditions to subclone scFv from pPNL6 into pPNL9 from plasmid or colony
Primers: (note,pPNL9 reverse has a 1 base mismatch T to G noted in bold below. This still allows amplification from pPNL6 that has scFv cloned into it…not the parental vector without scFv, and changes a lysine to a glutamine to remove potential cryptic proteolytic cleavage site.)
PNL9for: GACGTTCCAG ACTACGCTGG TGGTGGTGGT TCTGCTA
PNL9rev: GGGTTAGGGA TAGGCTTACC CT**G**TTGTTCT AGAATTCCG

NOTE: It is critical to use a proofreading thermal stable polymerase (such HiFi Platinum Taq) for the amplification. This can be done using colony PCR if a 5°C lower annealing temperature is used.

PCR conditions to subclone scFv from pPNL6 into pPNL9			
	Stock Conc.	µL/reaction	Conc. in reaction
10 x Buffer	10x	5	
$MgSO^4$	50 mM	1.5	1.5 mM
dNTPs	10 mM	1	0.2 mM
H_2O		39-X	
Primers	10 µM each	2	0.2 mM each
Template		X	
HiFi Platinum Taq (Invitrogen)	5U/µL	0.5	
Cycle: 95°C 5 minutes, 95°C 30 seconds, 55°C 30 seconds, 68°C 30 seconds, repeat 30 cycles, 68°C 5 minutes			

D) Mutagenic PCR
These conditions are used to amplify a specific insert and incorporate random point mutations. The mutagenized amplicon is subsequently cloned by "Gap Repair" into linearized pPNL6 to create a mutagenized library of the clone (10^6-10^7 diverse). Screening for improved affinity clones is discussed under "affinity maturation".

5 µL 10x Taq Buffer
5 µL 2 mM dNTP, 1 mM ATP (Final concentration in rx. 0.2 and 0.1mM)
2.5 µL Primer #1= PNL-6rev (10 µM, final 0.5 µM)
2.5 µL Primer #2 = PNL-6 for (10 µM, final 0.5 µM)
1.5 µL 50 mM MgCl2 (1.5 mM final concentration)
1 µL Platinum Taq enzyme NOT HI FI (Final 2.5 U)
2.5 µL 4 mM MnSO4 (Final concentration 0.2 mM)
X template
31.5-x H_20
50 µL total volume

Cycle: 95°C 5 minutes, 95°C 15 seconds, 50°C 30 seconds, 72°C 60 seconds, repeat 39 cycles, 72°C 5 minutes

3.7.3 BstN1 Digest for "Fingerprint analysis" of scFv inserts:

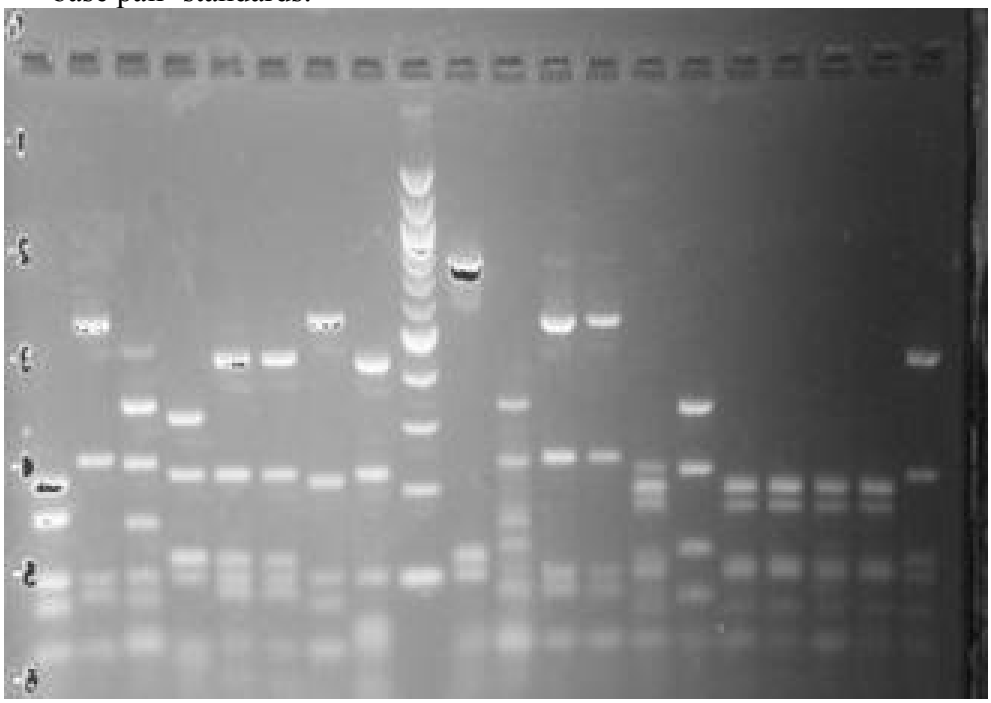
The digest uses amplicons from the PCR reaction using pPNL6 for and rev primers on either plasmid or colony PCR.

1. Use approximately 400ng of DNA straight form the PCR reaction into the BstN1 digest. This is usually about 20ul of the PCR reaction above.
   BstN1 Digestion:
   20ul of PCR amplicons
   2.5ul of 10x NEB Buffer #2
   0.25ul 100x BSA
   2.25ul of ddH_20
   25ul reaction

2. Incubate at 60°C for 1hour

3. Run all of it on a 2.5% NuSieve Gel containing Ethidium Bromide (25ul/L of 10mg/ml stock of EtBr) in the gel and using a sample loading buffer containing tartrazine dye for sample tracking and not the standard blue dyes. The blue dyes can often hide the faint bands of the digest making them harder to see.

4. Take a picture and analyze the different banding patterns to identify unique clones. Note: This analysis to identify unique or different clones is not recommended to identify different clones isolated from a mutagenic library. The figure below shows several different banding patterns indicating unique clones. The outside lanes are 50 base pair standards.



3.7.4 Gap Repair Cloning of scFv into Secretion Vector or Creating Mutagenized Libraries for Molecular Evolution

The standard LiAc TRAFCO yeast transformation method is used, as can be found at http://www.umanitoba.ca/faculties/medicine/biochem/gietz/Trafo.html

1. Prepare vector for cloning by linearization by double-restriction digesting:
   pPNL6 = Not1/Nhe1
   pPNL9 = Not1/Sfi1
2. Treat digest with phenol chloroform isoamyl alcohol and ethanol precipitate vector. Resuspend in water. This removes Sfi1 and does not precipitate the insert.
3. PCR-amplify scFv insert (PCR = Platinum Taq HiFi or mutagenic = non-proofreading Taq) from pPNL6-based clone using the following primers:
   pPNL6 cloning =PNL6 for/rev under mutagenic conditions to create sublibrary
   pPNL9 cloning = PNL9 for/rev for subcloning into secretion vector from pPNL6 surface expressed clone.

   Note: PCR amplification with PNL9 for/rev primers works best with either purified plasmid or a PCR template generated with pNL6for/rev primers. Use a 1/1000 dilution of the amplicon from the PNL6for/rev PCR reaction for template in the PNL9for/rev PCR.
4. Transform yeast using standard LiAc TRAFCO methodology. Mix linearized vector and PCR-amplified insert at a 1:3 Vector:Insert molar ratio (1:10 gives greater number of Transformants). Transform a linearized vector only for a control.
5. Selective plates take 3 to 5 days for colonies to appear. If creating a library, grow in liquid culture, take an OD_{600} reading at the beginning, and set up a dilution plate to determine total number of transformants. The starting liquid culture should be below 0.5 OD/mL and the OD_{600} should be checked daily until 5 to 10 OD/mL is reached, usually in 3 days. Subculture a 10x representation of the library diversity and expand 100-fold by growth in selective non-inducing media.
6. Freeze down several 10x aliquots of library in 30% glycerol and store at -80^{o}C. NOTE: We find our transformation frequency is very similar for "Gap Repair" and using super-coiled plasmid.

3.8 Secretion and Purification of scFv Antibodies

One of the appeals with recombinant antibodies is the ability to apply standard molecular manipulations to the antibody gene of interest. This allows researchers to move the isolated scFv gene into a variety of expression vectors allowing a wide spectrum of purification tags and organisms (*E. coli*, *S. cerevisiae*, *Pichia pastoris*, insect and mammalian) can be used.

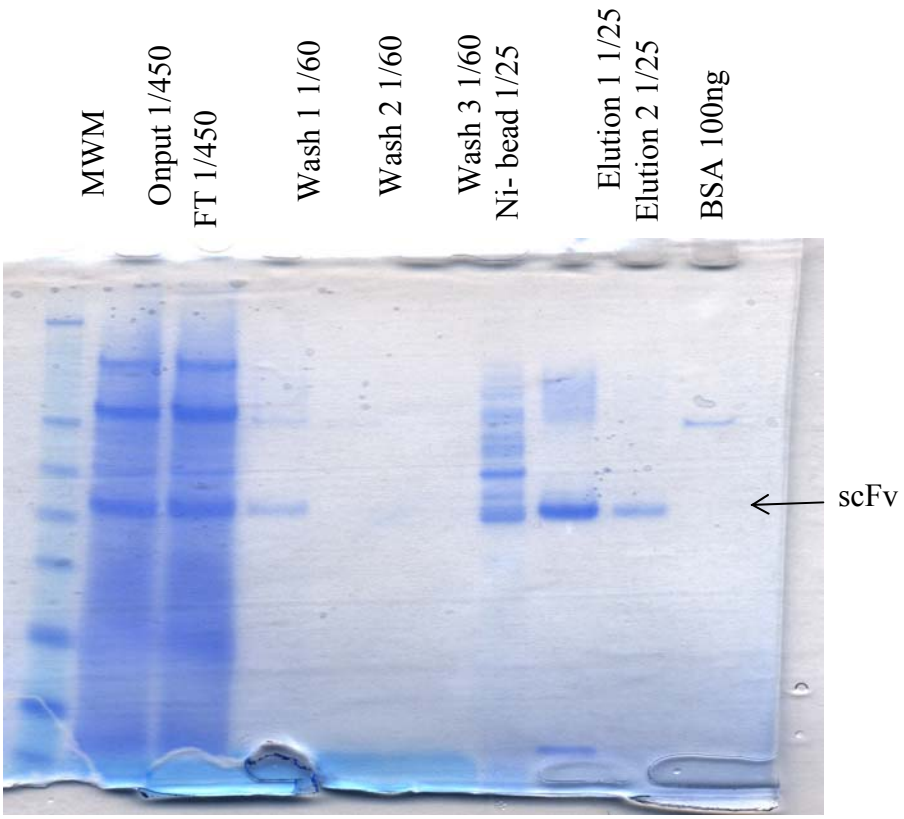
The protocol listed below is to be used with scFv transferred into the pPNL9 secretion vector using *S. cerevisiae* YVH10 cells for purification through the 6His tag.

Inoculate 50 mL SD+CAA + Trp. Culture at 30°C until saturated (o/n OD >3/mL)
Spin cells and resuspend in 50 mL YEP G/R containing antibiotic, 0.1% dex.
Induce 20° to 30° (check for optimal production) for 16 to 72 hours
Check OD
Check for bacterial contamination
Check pH of supernatant
Because culture supernatant contains ingredients that prohibit 6xHis – IMAC purification, do the following:
Spin cells – transfer supernatant to new tube
Adjust supernatant pH to ~7 with 10x NaH_2PO_4 pH 7.6
Dialyze or use microconcentrator to exchange buffer into 1x PBS (ideally want 6000x change in buffer)
Optimize supernatant before IMAC purification
0.5 M NaCl and 0.05% Tween 20
Remove 500 µL aliquot for analysis (onput fraction)
Add Qiagen Ni-NTA agarose beads to supernatant (capacity 0.5 to 1 mg/mL bead slurry): typically use 200 µL slurry/purification
Mix 4°C for 2 hours
Spin agarose beads 3000 RPM for 3 to 5 minutes
Remove 500 µL aliquot (flow through fraction)
Wash: 10 mL (0.5 M NaCl - 0.05% Tween 20 - pH 7 with NaH_2PO_4)
Mix 5 to 10 minutes at 4°C.
Spin 3000 RPM
Transfer supernatant to new tube (keep until gel analysis)
Remove 500 µL aliquot (wash fraction)
Elution
250 µL elution buffer -2xPBS (300 mM NaCl) – 200 mM imidiazole
Mix 20 to 30 minutes at 4°
Spin: transfer eluted scFv supernatant to new tube using 20 gauge syringe
Repeat for 2 to 4 total elution fractions
Keep beads for analysis
Analysis
Concentrate onput, FT, and wash fractions 10x with microcon
Visualize purification/purity with 4 to 12% Bis-Tris gel (Invitrogen)
Pool appropriate elution fractions and dialyze into 1x PBS to remove imidiazole (typically 2x 0.5 mL scFv to 2 L PBS)

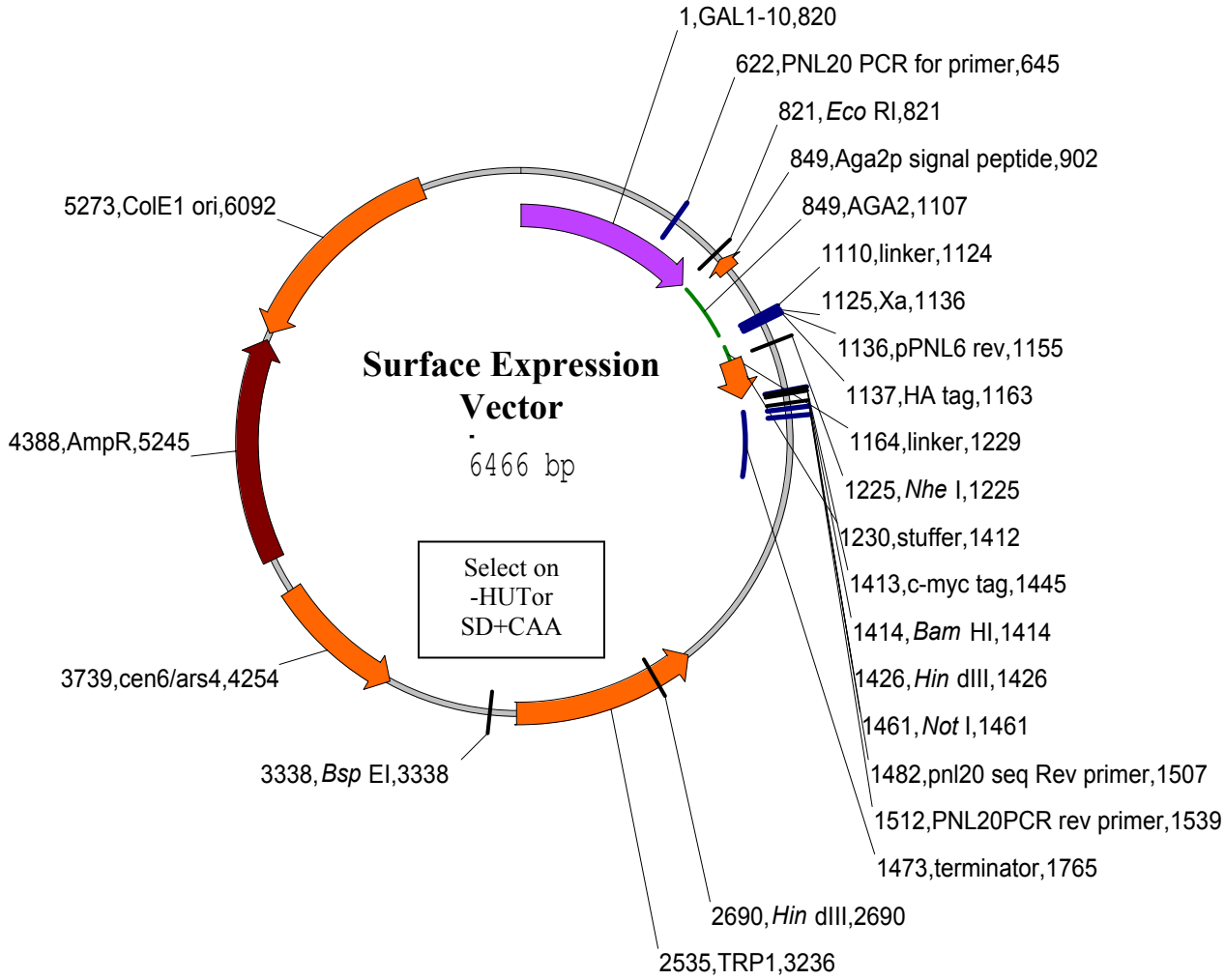
Quantify purified fraction

We generally obtain >100 µg purified scFv/50 mL induction culture = 2-10 mg/L yield.

Figure 8. SDS-Page of 6-His purified scFv.

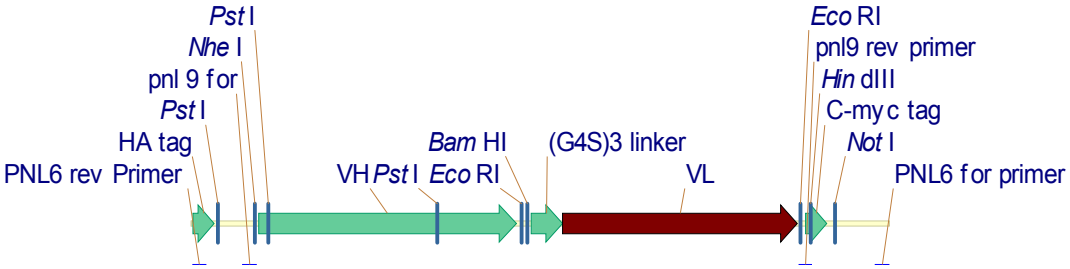


Plasmids, Strains, and Growth Conditions



EBY100= (Leu$^-$, Trp$^-$) BJ5465 is MATa. It has the auxotrophic: ura3-52 (a Ty element insertion with no detectable background reversion frequency), trp1 (an amber point mutation), leu2δ200, his3δ200, pep4:HIS3, prbd1.6R, can1, GAL. EBY100 has genomic insertion of AGA1 regulated by GAL promoter with a URA3 selectable marker. The scFv library is in this strain.

Growth
EBY100 with pPNL6 would be grown on in/on SD +CAA (-Ura -Trp)
EBY100 without a plasmid: add Trp to SD+CAA or YPD.



user manual scFv surface construct in pPNL6

990 bp

```
                                                PstI
                                               ~~~~~~
      R  Y  P   Y  D  V  P   D  Y  A   L  Q  A   S  G  G  G ·
1     TAGATACCCA TACGACGTTC CAGACTACGC TCTGCAGGCT AGTGGTGGTG
      ATCTATGGGT ATGCTGCAAG GTCTGATGCG AGACGTCCGA TCACCACCAC
                                                  NheI
                                                 ~~~~~~~
      · G  S  G   G  G  G   S  G  G  G   G  S  A   S  K  V
51    GTGGTTCTGG TGGTGGTGGT TCTGGTGGTG GTGGTTCTGC TAGCAAGGTA
      CACCAAGACC ACCACCACCA AGACCACCAC CACCAAGACG ATCGTTCCAT
          PstI
         ~~~~~~
       Q  L  Q  Q   S  G  P   G  L  V   K  P  S  Q   T  L  S ·
101   CAGCTGCAGC AGTCAGGTCC AGGACTGGTG AAGCCCTCGC AGACCCTCTC
      GTCGACGTCG TCAGTCCAGG TCCTGACCAC TTCGGGAGCG TCTGGGAGAG
      · L  T  C   G  F  S  G   D  S  F   S  N  N   N  V  A  W ·
151   ACTCACCTGT GGCTTCTCCG GGGACAGTTT CTCCAACAAC AATGTTGCTT
      TGAGTGGACA CCGAAGAGGC CCCTGTCAAA GAGGTTGTTG TTACAACGAA
      · N  W  I   R  Q  S   P  S  R  G   L  E  W   L  G  R
201   GGAACTGGAT CAGGCAGTCC CCGTCGAGAG GCCTTGAGTG GCTGGGAAGG
      CCTTGACCTA GTCCGTCAGG GGCAGCTCTC CGGAACTCAC CGACCCTTCC
       T  Y  R  G   S  K  W   Y  N  D   Y  A  E  S   V  R  G ·
251   ACATACCGCG GGTCCAAGTG GTATAATGAT TATGCAGAGT CTGTGAGAGG
      TGTATGGCGC CCAGGTTCAC CATATTACTA ATACGTCTCA GACACTCTCC
                                                    PstI
                                                   ~~~~~~
      · R  I  T   I  N  A  D   T  S  K   N  Q  F   S  L  Q  L ·
301   TCGAATAACC ATCAACGCAG ACACATCCAA GAACCAGTTC TCCCTGCAGC
      AGCTTATTGG TAGTTGCGTC TGTGTAGGTT CTTGGTCAAG AGGGACGTCG
      · N  S  V   T  P  E   D  T  A  V   Y  Y  C   A  R  G
351   TGAACTCTGT GACTCCCGAG GACACGGCTG TCTATTATTG TGCAAGAGGG
      ACTTGAGACA CTGAGGGCTC CTGTGCCGAC AGATAATAAC ACGTTCTCCC
       F  S  T  S   V  G  Y   Y  F  Q   H  W  G  Q   G  T  L ·
401   TTTAGTACTT CGGTGGGTTA TTACTTCCAG CACTGGGGCC AGGGTACCCT
      AAATCATGAA GCCACCCAAT AATGAAGGTC GTGACCCCGG TCCCATGGGA
                        EcoRI    BamHI
                       ~~~~~~~  ~~~~~~~
      · V  T  V   S  S  G  I   L  G  S   G  G  G   G  S  G  G ·
451   GGTCACCGTC TCCTCAGGAA TTCTAGGATC CGGTGGCGGT GGCAGCGGCG
      CCAGTGGCAG AGGAGTCCTT AAGATCCTAG GCCACCGCCA CCGTCGCCGC
      · G  G  S   G  G  G   G  S  Q  P   V  L  T   Q  S  P
501   GTGGTGGTTC CGGAGGCGGC GGTTCTCAGC CTGTGCTGAC TCAGTCACCC
      CACCACCAAG GCCTCCGCCG CCAAGAGTCG GACACGACTG AGTCAGTGGG
       S  V  S  G   T  P  G   Q  R  V   T  I  S  C   S  G  S ·
```

```
551   TCAGTGTCTG GGACCCCCGG GCAGAGGGTC ACCATCTCTT GTTCTGGAAG
      AGTCACAGAC CCTGGGGGCC CGTCTCCCAG TGGTAGAGAA CAAGACCTTC
      · S  S  N   I  G  N  N   H  V  Y   W  Y  Q   Q  L  P  G ·
601   CAGCTCCAAC ATCGGAAATA ATCATGTTTA CTGGTACCAG CAACTCCCAG
      GTCGAGGTTG TAGCCTTTAT TAGTACAAAT GACCATGGTC GTTGAGGGTC
      ·  T  A  P   K  L  L   I  Y  R  D   N  R  R   L  S  G
651   GAACGGCCCC CAAACTCCTC ATCTATAGGG ATAATCGGCG GCTCTCAGGG
      CTTGCCGGGG GTTTGAGGAG TAGATATCCC TATTAGCCGC CGAGAGTCCC
       V  P  D  R   F  S  G   S  K  S   G  T  S  A   S  L  A ·
701   GTCCCTGACC GATTCTCTGG CTCCAAGTCG GGCACCTCAG CCTCCCTGGC
      CAGGGACTGG CTAAGAGACC GAGGTTCAGC CCGTGGAGTC GGAGGGACCG
      · I  S  G   L  R  S  D   D  E  A   E  Y  F   C  A  A  W ·
751   CATCAGTGGG CTCCGGTCCG ACGATGAGGC TGAGTATTTC TGTGCAGCAT
      GTAGTCACCC GAGGCCAGGC TGCTACTCCG ACTCATAAAG ACACGTCGTA
      ·  D  A  S   L  S  G   L  Y  V  F   G  G  G   T  K  L
801   GGGATGCCAG CCTGAGTGGT CTCTACGTGT TCGGCGGAGG GACCAAGCTC
      CCCTACGGTC GGACTCACCA GAGATGCACA AGCCGCCTCC CTGGTTCGAG
                   EcoRI          HindIII
                   ~~~~~~         ~~~~~~~
       T  V  L  S   G  I  L   E  Q  K   L  I  S  E   E  D  L ·
851   ACCGTCCTAT CCGGAATTCT AGAACAAAAG CTTATTTCTG AAGAAGACTT
      TGGCAGGATA GGCCTTAAGA TCTTGTTTTC GAATAAAGAC TTCTTCTGAA
                   NotI
                  ~~~~~~~~
      · *  *  L
901   GTAATAGCTC GGCGGCCGCA
      CATTATCGAG CCGCCGGCGT
```

PNL9rev primer = GCCTTAAGAT CTTGTTTTCG

Note: PNL9rev primer will only hybridize to an scFv clone in the library vector pPNL6 and not the starting vector.

Sequence of pPNL6 and pPNL9

pPNL6 sequence:

cgacaggttatcagcaacaacacagtcatatccattctcaattagctctaccacagtgtgtgaaccaatgtatccagcaccacctgt
aaccaaaacaattttagaagtactttcactttgtaactgagctgtcatttatattgaattttcaaaaattcttactttttttttggatggacgc
aaagaagtttaataatcatattacatggcattaccaccatatacatatccatatacatatccatatctaatcttacttatatgttgtggaaa
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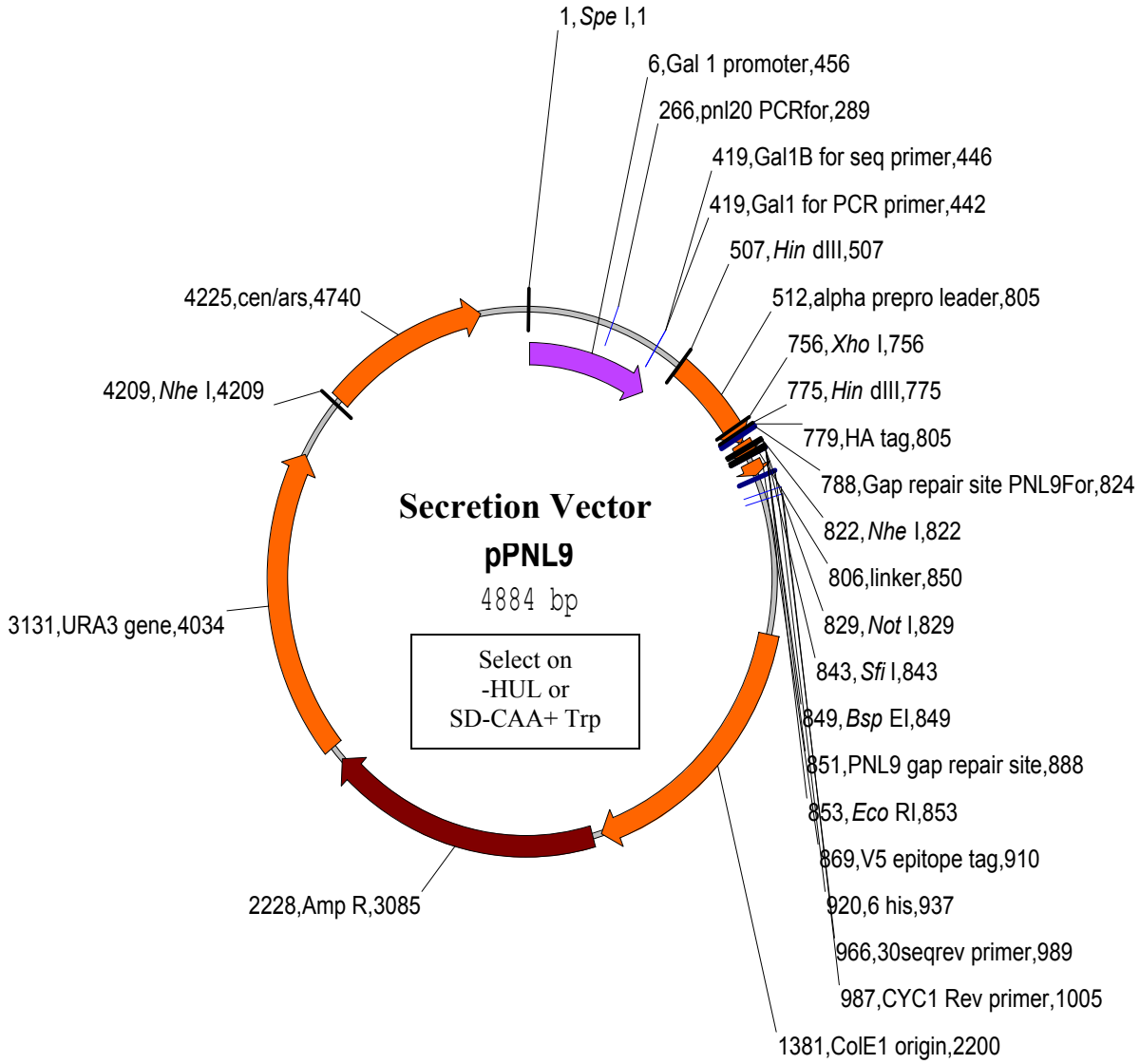
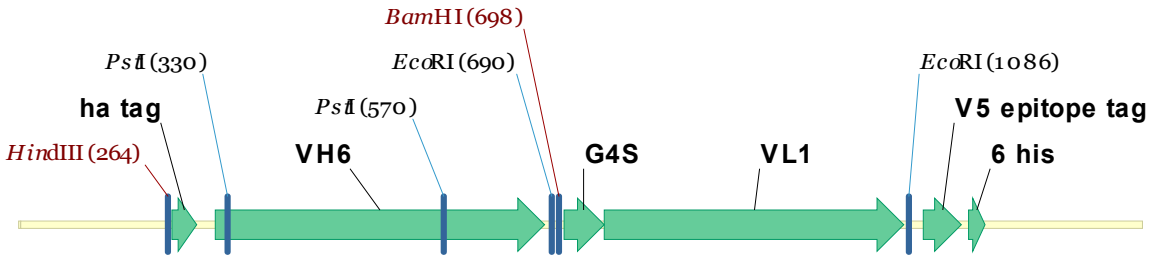


YVH10 (Ura-, Trp-) is BJ5464 and is MAT-alpha. The other markers are: ura3-52 (a Ty element insertion with no detectable background reversion frequency), trp1 (an amber point mutation), leu2δ200, his3δ200, pep4:HIS3, prbd1.6R, can1, GAL.
YVH10 has the yeast PDI gene integrated in tandem with the endogenous copy and is under the constitutive glyceraldehydes (GADPH) promoter with Leu2 as a selectable marker. This strain is used to secrete and purify the scFv.

Growth
YVH10 with pPNL9 should be selected on SD + CAA + Trp
YVH10 strain without a plasmid should be grown on: The media (SD +CAA) must contain TRP and URA. It can also be grown on YPD.



Fragment of example of scFv in secretion vector

1246 bp (molecule 5628 bp)

```
100   CCGGCTGAAG CTGTCATCGG TTACTCAGAT TTAGAAGGGG ATTTCGATGT
      TGCTGTTTTG CCATTTTCCA ACAGCACAAA TAACGGGTTA TTGTTTATAA
      GGCCGACTTC GACAGTAGCC AATGAGTCTA AATCTTCCCC TAAAGCTACA
      ACGACAAAAC GGTAAAAGGT TGTCGTGTTT ATTGCCCAAT AACAAATATT
      HindIII
      ~~~~~~
200   ATACTACTAT TGCCAGCATT GCTGCTAAAG AAGAAGGGGT ATCTCTCGAG
      AAAAGAGAGG CTGAAGCTTA CCCATACGAC GTTCCAGACT ACGCTGGTGG
      TATGATGATA ACGGTCGTAA CGACGATTTC TTCTTCCCCA TAGAGAGCTC
      TTTTCTCTCC GACTTCGAAT GGGTATGCTG CAAGGTCTGA TGCGACCACC
                                  PstI
                                 ~~~~~~~
300   TGGTGGTTCT GCTAGCAAGG TACAGCTGCA GCAGTCAGGT CCAGGACTGG
      TGAAGCCCTC GCAGACCCTC TCACTCACCT GTGGCTTCTC CGGGGACAGT
      ACCACCAAGA CGATCGTTCC ATGTCGACGT CGTCAGTCCA GGTCCTGACC
      ACTTCGGGAG CGTCTGGGAG AGTGAGTGGA CACCGAAGAG GCCCCTGTCA
400   TTCTCCAACA ACAATGTTGC TTGGAACTGG ATCAGGCAGT CCCCGTCGAG
      AGGCCTTGAG TGGCTGGGAA GGACATACCG CGGGTCCAAG TGGTATAATG
      AAGAGGTTGT TGTTACAACG AACCTTGACC TAGTCCGTCA GGGGCAGCTC
      TCCGGAACTC ACCGACCCTT CCTGTATGGC GCCCAGGTTC ACCATATTAC
      PstI
      ~~~~~~~
500   ATTATGCAGA GTCTGTGAGA GGTCGAATAA CCATCAACGC AGACACATCC
      AAGAACCAGT TCTCCCTGCA GCTGAACTCT GTGACTCCCG AGGACACGGC
      TAATACGTCT CAGACACTCT CCAGCTTATT GGTAGTTGCG TCTGTGTAGG
      TTCTTGGTCA AGAGGGACGT CGACTTGAGA CACTGAGGGC TCCTGTGCCG
      EcoRI
      ~~~~~~~
      BamHI
      ~~~
600   TGTCTATTAT TGTGCAAGAG GGTTTAGTAC TTCGGTGGGT TATTACTTCC
      AGCACTGGGG CCAGGGTACC CTGGTCACCG TCTCCTCAGG AATTCTAGGA
      ACAGATAATA ACACGTTCTC CCAAATCATG AAGCCACCCA ATAATGAAGG
      TCGTGACCCC GGTCCCATGG GACCAGTGGC AGAGGAGTCC TTAAGATCCT
      BamHI
      ~~~
700   TCCGGTGGCG GTGGCAGCGG CGGTGGTGGT TCCGGAGGCG GCGGTTCTCA
      GCCTGTGCTG ACTCAGTCAC CCTCAGTGTC TGGGACCCCC GGGCAGAGGG
      AGGCCACCGC CACCGTCGCC GCCACCACCA AGGCCTCCGC CGCCAAGAGT
      CGGACACGAC TGAGTCAGTG GGAGTCACAG ACCCTGGGGG CCCGTCTCCC
800   TCACCATCTC TTGTTCTGGA AGCAGCTCCA ACATCGGAAA TAATCATGTT
      TACTGGTACC AGCAACTCCC AGGAACGGCC CCCAAACTCC TCATCTATAG
```

```
        AGTGGTAGAG AACAAGACCT TCGTCGAGGT TGTAGCCTTT ATTAGTACAA
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 900    GGATAATCGG CGGCTCTCAG GGGTCCCTGA CCGATTCTCT GGCTCCAAGT
        CGGGCACCTC AGCCTCCCTG GCCATCAGTG GGCTCCGGTC CGACGATGAG
        CCTATTAGCC GCCGAGAGTC CCCAGGGACT GGCTAAGAGA CCGAGGTTCA
        GCCCGTGGAG TCGGAGGGAC CGGTAGTCAC CCGAGGCCAG GCTGCTACTC
        EcoRI
        ~~~~~~~
1000    GCTGAGTATT TCTGTGCAGC ATGGGATGCC AGCCTGAGTG GTCTCTACGT
        GTTCGGCGGA GGGACCAAGC TCACCGTCCT ATCCGGAATT CTAGAACAAC
        CGACTCATAA AGACACGTCG TACCCTACGG TCGGACTCAC CAGAGATGCA
        CAAGCCGCCT CCCTGGTTCG AGTGGCAGGA TAGGCCTTAA GATCTTGTTG
1100    AGGGTAAGCC TATCCCTAAC CCTCTCCTCG GTCTCGATTC TACGCGTACC
        GGTCATCATC ACCATCACCA TTGAGTTTAA ACCCGCTGAT CCTAGAGGGC
        TCCCATTCGG ATAGGGATTG GGAGAGGAGC CAGAGCTAAG ATGCGCATGG
        CCAGTAGTAG TGGTAGTGGT AACTCAAATT TGGGCGACTA GGATCTCCCG
1200    CGCATCATGT AATTAGTTAT GTCACGCTTA CATTCACGCC CTCCCCCCAC
        ATCCGCTCTA ACCGAAAAGG AAGGAGTTAG ACAACCTGAA GTCTAGGTCC
        GCGTAGTACA TTAATCAATA CAGTGCGAAT GTAAGTGCGG GAGGGGGGTG
        TAGGCGAGAT TGGCTTTTCC TTCCTCAATC TGTTGGACTT CAGATCCAGG
1300    CTATTTATTT TTTTATAGTT ATGTTAGTAT TAAGAACGTT ATTTAT
        GATAAATAAA AAAATATCAA TACAATCATA ATTCTTGCAA TAAATA
```

pPNL9 sequence:

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Frequently asked questions "FAQS"

These are questions and answers that are also posted on the web page (http://www.biomolecular.org/resources/index.html). You should check in periodically to check for updates and revisions.

1. Some proteins we use seem to stick to all the yeast. Why?
   A: Before starting any selection we routinely check the protein of interest for binding to a single scFv clone that is not induced (the control sent with the library), the library not induced and the library in the induced state. You should see no labeling in any case. If there is labeling it could be binding to the yeast surface or the scFv in a nonspecific fashion. We have the most reproducible results and experience with peptides and secreted proteins. However, we have also obtained antigen specific scFv to a variety of cytoplasmic proteins. We recommend trying different selection buffers to decrease or eliminate the nonspecific binding. For example, 0.1% tween/PBS, No EDTA, etc…

2. I have gone through five rounds of selection and all I got were secondary reagents binders or nothing. What's wrong?
   A: It could be a large number of possibilities. The most common reasons are listed below.
   - Did you check that the sorter was set up properly? Did the flow cytometer operator show you what was sorted by rerunning that sample or doing a test sort of labeled beads? Did you set up a dilution plate from the sorted cells? Was the number what you expected? We generally see about a 40-70% plating efficiency from the number of cells sorted.
   - Do you know your antigen is biotinylated? How? We use the Pierce HABA methodology to determine moles of biotin per mole of antigen and strive for a 1-2 moles of biotin /mole of antigen.
   - Try increasing the concentration of antigen up to 1uM.
   - There is also the possibility there are no binders to your protein.
   - Note, we have never <u>not</u> obtained a streptavidin binder if we go through five rounds of selection.
   - You may want to try a test selection using Biotinylated EGF. The quality of the aliquot of the library you received was checked by doing a multiplex screen using biotinylated EGF and f3 other peptide antigens. Binders were obtained for all. We have repeated this QC at least twice. No, we will not provide you the EGF.

3. Can I just use flow cytometric sorting or just use magnetic beads to get my Antigen specific clones?
   A: Yes, however we believe combining the two is the most powerful way to screen the complete diversity of the library. In our paper, HEL was isolated using the Macs system only.

4. I would like to do a test selection on the library with an antigen I know will work. What do you suggest?
   A: Hen Egg lysozyme (HEL) was used as test antigen by Dane Wittrups group and 3 high affinity scFv clones were identified. HEL is cheap and is easily biotinylated. I do not recommend using the 378p peptide that was included as a control, as we did not include enough antigen for an entire selection. We have used a number of types antigens that are published in the Nature Biotech paper, any of these would work well.

Changes to this revision from 030305:

1. The following "note" was added to primers section on page 27:
   PCR conditions to subclone scFv from pPNL6 into pPNL9 from plasmid or colony

Primers: (note, pPNL9 reverse has a 1 base mismatch T to G noted in bold below. This still allows amplification from pPNL6 with and scFv present and changes a lysine to a glutamine to remove potential cryptic proteolytic cleavage site.)

***One should note that these primers will not hybridize to the pPNL6 parental vector but only to the pPNL6 with scFv insert.**

PNL9for: GACGTTCCAG ACTACGCTGG TGGTGGTGGT TCTGCTA

PNL9rev: GGGTTAGGGA TAGGCTTACC CT**G**TTGTTCT AGAATTCCG

An additional vector diagram is included that showing an scFv clone from the library with all relevant primers and restriction sites as well as the surrounding sequence in both the secretion vector and surface expression vector.

Changes to Revision 031113:

1. Try to more clearly indicate the need to duplicate the data in Figures 6 and 7 with the control scFv clone included in the kit. Generating a data set as in Figure 4 might also be useful.

2. Try to more clearly indicate the need to duplicate the data for HA and myc staining of the library in Figure2.